Role of the β1-integrin cytoplasmic tail in mediating invasin-promoted internalization of *Yersinia*

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Accepted 18 April 2002
Journal of Cell Science 115, 2669-2678 (2002) © The Company of Biologists Ltd

Summary

Invasin of *Yersinia pseudotuberculosis* binds to β1-integrins on host cells and triggers internalization of the bacterium. To elucidate the mechanism behind the β1-integrin-mediated internalization of *Yersinia*, a β1-integrin-deficient cell line, GD25, transfected with wild-type β1A, β1B or different mutants of the β1A subunit was used. Both β1A and β1B bound to invasin-expressing bacteria, but only β1A was able to mediate internalization of the bacteria. The cytoplasmic region of β1A, differing from β1B, contains two NPXY motifs surrounding a double threonine site. Exchanging the tyrosines of the two NPXYs to phenylalanines did not inhibit the uptake, whereas a marked reduction was seen when the first tyrosine (Y783) was exchanged to alanine. A similar reduction was seen when the two nearby threonines (TT788-9) were exchanged with alanines. It was also noted that cells affected in bacterial internalization exhibited reduced spreading capability when seeded onto invasin, suggesting a correlation between the internalization of invasin-expressing bacteria and invasin-induced spreading. Likewise, integrins defective in forming peripheral focal complex structures was unable to mediate uptake of invasin-expressing bacteria.

Key words: Invasin, β1-integrin, *Yersinia pseudotuberculosis*, Focal complexes, Bacterial internalization

Introduction

Integrins are a large family of transmembrane αβ heterodimeric receptors that mediate cell-cell and cell-matrix interactions (Hynes, 1992; van der Flier and Sonnenberg, 2001). They play vital roles in embryonic development and are central players in several cellular functions, including cell spreading and migration (Fässler et al., 1996; Brakebusch et al., 1997; Sheppard, 2000; Stupack et al., 2000). Binding of integrins to extracellular matrix (ECM) proteins induces formation of focal adhesion structures. These are sites where the cellular actin cytoskeleton is connected to the integrins via a protein complex containing a variety of signaling and actin binding proteins, including focal adhesion kinase (FAK), Src, Crk-associated substrate (p130Cas), α-actinin, vinculin, paxillin and talin (Burridge et al., 1990; Giancotti and Ruoslahti, 1999; Liu et al., 2000). The integrin receptors, which are influenced both from the external environment and from intracellular signals, mediate both outside-in and inside-out signal transduction (Schwartz et al., 1995). The cytoplasmic tail is vital for integrin function, and integrins lacking the cytoplasmic part exhibit reduced ligand-binding activity, impaired activation of downstream signaling molecules and do not localize to focal adhesion structures (LaFlamme et al., 1997). There are five splice variants of the human β1-integrin, β1A, β1B, β1C-1, β1C-2 and β1D, differing in the end of the cytoplasmic domain (Armulik, 2002). The β1A- and β1D-integrins localize to matrix-associated focal adhesions, and ligation triggers tyrosine phosphorylation of cytoplasmic proteins, whereas the β1B, β1C-1 and β1C-2 variants are negative for this. β1B is also inhibitory to β1A and β1D when overexpressed (de Melker and Sonnenberg, 1999; Armulik, 2002). The cytoplasmic part of β1A and β1D contains two NPXY motifs and in addition β1A also has a double threonine site between these NPXY motifs. The corresponding part of β1B has a completely different sequence lacking known motifs, whereas the β1C variants are parts of an Alu element (Armulik, 2002).

In addition to binding ECM proteins such as fibronectin and laminin, the β1A subunit, dimerized with α3, α4, α5, α6 or αv, also binds to the bacterial adhesin, invasin, which is expressed on enteropathogenic *Yersinia* species (Isberg and Leong, 1990; Leong et al., 1990; Rankin et al., 1992). Interaction of invasin with integrins results in internalization of the bacteria, whereas binding to matrix-protein-coated bacteria or beads usually does not (Tran Van Nhieu and Isberg, 1991). This difference is thought to be caused by the high-affinity binding of integrins to invasin and maybe also result from qualitative differences in signal transduction. Invasin, which bind with about 100-fold higher affinity than fibronectin to integrins, can efficiently compete out fibronectin from integrin binding (Tran Van Nhieu and Isberg, 1991). In addition, invasin expressed by *Y. pseudotuberculosis* contains a central dimerization region enabling multimerization of the integrin receptor that greatly contributes to efficient internalization of bacteria (Dersch and Isberg, 1999). The crystal structures of the integrin-binding regions of invasin and

fibronectin showed that these two ligands recognize similar residues on the integrin, although invasin lacks an alanine-glycine-asparagine (RGD) sequence and the overall contour was different (Hamburger et al., 1999). The higher affinity seen for invasin is probably due to the involvement of more hydrogen bonds in the binding to the integrin and also to a less flexible interdomain region on invasin (Leahy et al., 1996; Hamburger et al., 1999).

β1-integrin-mediated phagocytosis of invasin-expressing bacteria is an actin-dependent process involving local membrane protrusions. It also requires tyrosine kinase activity, and earlier studies have implicated the tyrosine kinases Src and FAK as well as the small G-protein Rac as important players in this signaling (Alrutz and Isberg, 1998; McGee et al., 2001). However, pathogenic *Yersinia* strains can actively block the uptake process by injecting antiphagocytic factors into host cells (Rosqvist et al., 1988a; Persson et al., 1997). The antiphagocytic effect has been attributed to two *Yersinia* outer proteins, YopE and YopH, which are delivered into host cells through a type III secretion mechanism (Cornelis et al., 1998). YopE is a GTPase-activating protein acting on Rho family proteins (Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000). YopH is a protein tyrosine phosphatase that recognizes focal complex structures and dephosphorylates p130Cas, paxillin and FAK, which all are proteins implicated in β1-integrin signaling and focal complex dynamics. The effect of the dephosphorylation by YopH is that the focal contacts are disrupted and bacterial uptake by cells is blocked (Black and Bliska, 1997; Persson et al., 1997). The YopH protein contains a sequence that mediates its localization to integrin-containing focal complex structures (Persson et al., 1999). Interestingly, mutations that abolish YopH colocalization with these structures strongly reduce YopH's capacity to block phagocytosis (Persson et al., 1999), suggesting that integrin-associated focal complex structures are important for β1-integrin-mediated uptake of bacteria.

The aim of this study was to identify features of the β1-integrin that are important for invasin-promoted phagocytosis. For this purpose we took advantage of the fibroblast-like cell line GD25, which was derived from β1-integrin-deficient embryonic stem cells (Fässler et al., 1995; Wennerberg et al., 1996). The GD25 cells and stable transfectants of these, expressing wild-type and mutated variants of the β1-integrin, were used to investigate the mechanism behind invasin-promoted bacterial internalization. We found that invasin, in contrast to fibronectin, can bind to inactive β1B-integrin but without triggering uptake of bacteria. The first NPXY motif and the double threonine site in the β1A cytoplasmic chain were found to be of importance for internalization of bacteria, but there was no requirement for tyrosine phosphorylation of the β1-integrin cytoplasmic NPXY motifs. There were also a correlation between integrin-mediated internalization of invasin-expressing bacteria and invasin-induced spreading.

Materials and Methods

Proteins, peptides and antibodies

The C-terminal region of invasin of *Y. pseudotuberculosis* (amino acids 487-986) was cloned into the pGEX vector (GST-invΔ1) and transformed into the BL21 strain of *Echerichia coli*. The GST and GST-invΔ1 proteins were purified according to the protocol provided by the manufacturer (Amersham Biosciences). The glycine arginine glycine aspartic acid serine (GRGDS) peptide was purchased from Bachem Feinchemicalien AG, Germany. Rabbit anti-*Yersinia* serum was kindly provided by R. Rosqvist (Rosqvist et al., 1988b), and the goat serum was from Agrisera. The following antibodies were purchased: Armenian hamster anti-mouse β1 (clone HMβ1-1; Pharmingen), mouse anti-phosphotyrosine (PY20), horseradish peroxidase (HRP)-conjugated mouse anti-PY20, mouse anti-paxillin, mouse anti-FAK (clone 77), mouse anti-p130Cas (BD Transduction laboratories), mouse anti-human vinculin (Sigma-Aldrich), normal donkey serum, fluorescein (FITC)-labeled goat anti-Armenian hamster, lissamin-rhodamine (LRSC)-labeled donkey anti-mouse, peroxidase-conjugated donkey anti-rabbit, (Jacksson ImmunoResearch Laboratories Inc.), peroxidase-conjugated sheep anti-mouse (Amersham Pharmacia Biotech), FITC-labeled swine anti-rabbit and rhodamine (TRITC)-labeled swine anti-rabbit (DAKO A/S).

Cell culture

The GD25 cell line lacks the β1-integrins owing to disruption of the *β1* gene (Fässler et al., 1995); all the other cell lines were derived from GD25 by stable transfection with cDNA encoding wild-type β1-integrin or mutated β1 subunit. The cell lines GD25β1A, GD25β1B, GD25β1AY795F, GD25β1AY783F, GD25β1AYY783/795FF and GD25β1ATT788-9AA have been described previously (Wennerberg et al., 1996; Sakai et al., 1998; Wennerberg et al., 1998; Armulik et al., 2000). The GD25β1Y783A, GD25β1AT788A, GD25β1AT789A and GD25β1AD130A lines where generated as previously described for the GD25β1ATT788-9AA cell line (Wennerberg et al., 1998). The GD25 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactive fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). The transfected cells were grown in the same medium supplemented with puromycin (20 μg/ml).

Fluorescence activated cell sorter (FACS) analysis

Cells (1×10^6) were trypsinized and washed twice with fresh medium. Subsequently, the cells were suspended in primary antibody (HMβ1-1, 1:100) in FACS-PBS (phosphate buffered saline containing 10% FCS and 0.001% NaN_3) and incubated on ice for 20 minutes. After washing, the cells were incubated with secondary antibody (FITC-conjugated goat anti-Armenian hamster antibody, 1:60) for 20 minutes in FACS-PBS, washed cells were resuspended in FACS-PBS and analyzed (10,000 cells per sample) using a FACScan® (BD Biosciences).

Bacterial uptake

The multiple Yop mutant strain (MYM) lacking YadA, YopM, E, K, H and YpkA of *Yersinia pseudotuberculosis* (Håkansson et al., 1996) was used to investigate bacterial uptake into the cells. An over night culture of MYM, grown in Luria broth at 26°C, was diluted 1:120 in cell culture medium and incubated for 1 hour at 26°C. This culture was used to infect cells grown for 18 hours on cover slips, at a multiplicity of infection of 150:1. The infection was carried out for 30 minutes at 37°C in an atmosphere of 5% CO_2. The cells were then fixed in 2% paraformaldehyde and double immunofluorescence labeling was applied to distinguish between extracellular and total cell-associated bacteria (Heesemann and Laufs, 1985; Rosqvist et al., 1988a). The extracellularly localized bacteria were labeled by incubating the samples with rabbit anti-*Yersinia* serum (1:500). The cells were subsequently permeabilized in 0.5% Triton X-100, and extracellularly localized bacteria were stained by incubating with FITC-conjugated anti-rabbit antibodies (1:100). All cell-associated bacteria were then stained with anti-*Yersinia* serum (1:500) and

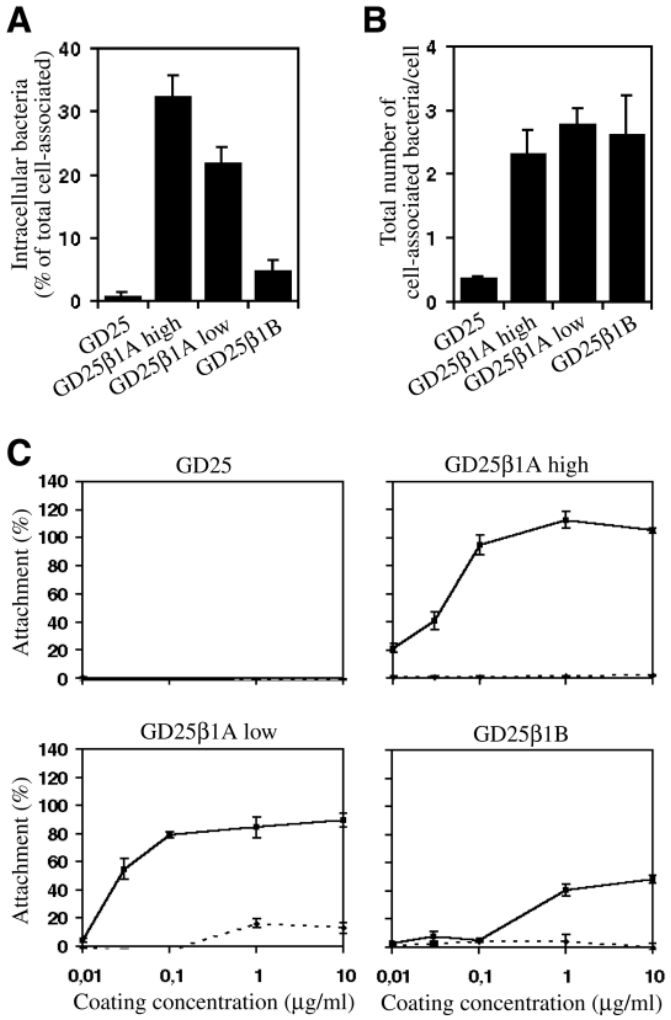


Fig. 1. β1-integrin-mediated internalization of *Yersinia*, and integrin attachment to invasin. Cells were infected with *Yersinia* at a calculated bacteria:cell ratio of 150:1 for 30 minutes at 37°C, and thereafter fixed and stained for extracellular and total cell-associated bacteria, respectively. (A) The percentage of cell-associated bacteria located intracellularly is shown. (B) The total number of bacteria associated to one cell is shown. The values represent the means±s.e.m. of at least five separate experiments. (C) The indicated cells were allowed to bind to microtiter plates coated with GST-invasin (solid line), GST alone (dotted line) or vitronectin for 1 hour. The number of cells adhering to vitronectin (coated at 10 μg/ml) represents 100% of attached cells.

LRSC-conjugated anti-rabbit serum (1:100). The cells were finally mounted using the Ultimate Mounting Media (UMM) (Fällman et al., 1995), and the specimens were analyzed in a fluorescence microscope (Zeiss axioskop 50), where 50 cells per slide were analyzed in at least five separate experiments.

Cell attachment assay

Cell attachment was quantified as previously described (Wennerberg et al., 1996). Briefly, 96-well plates were coated with vitronectin (10 μg/ml) or various concentrations of GST or GST-invΔ1 in PBS (overnight at 4°C), and thereafter blocked with 1% heat-treated bovine serum albumine (BSA) for 2 hours at 37°C. Cells were suspended in serum-free DMEM, plated to coated wells (1×10^5 cells/well) and allowed to attach for 1 hour at 37°C. Unattached cells were washed away, and the remaining cells were fixed in 96% ethanol and stained in crystal violet (0.1%) for 30 minutes. Excess stain was washed away with water and attached cells were dissolved in Triton X-100 in PBS (0.2%). The absorbance was read in a microtiter plate reader at 600 nm. All samples were analyzed in triplicates. The amount of cells of each cell line that were attached to wells coated with vitronectin (10 μg/ml) was set as 100% binding.

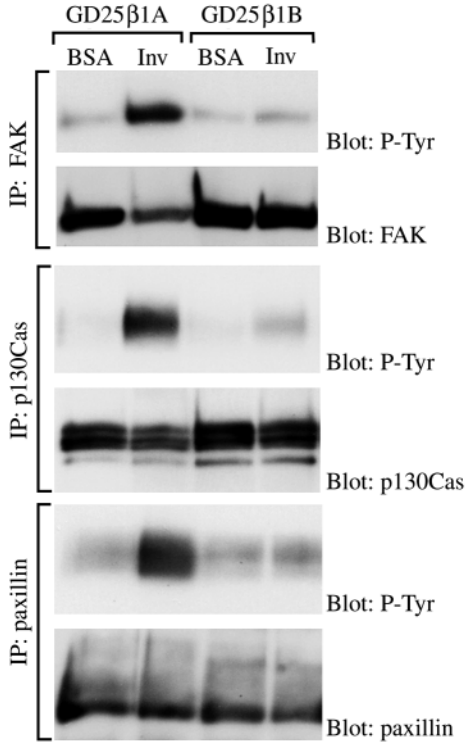


Fig. 2. Tyrosine phosphorylation of focal contact proteins. The GD25β1A and GD25β1B cell lines were allowed to attach to invasin for 60 minutes and then lysed in RIPA buffer. The lysates were immunoprecipitated for FAK, p130Cas or paxillin, and the precipitated proteins were analyzed by western blotting using antibodies specific for phosphotyrosine. As a loading control, the membranes were stripped and incubated with protein-specific antibodies.

Immunoprecipitation and western blotting

Serum-starved cells were trypsinized and treated with soybean trypsin inhibitor type II (Sigma-Aldrich, Stockholm, Sweden). Cells were washed once with serum-free DMEM and plated on dishes (6×10^6 cells) coated with GST-invasin (10 μg/ml) or BSA. The cells were allowed to attach at 37°C for 60 minutes and subsequently lysed in RIPA (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 200 μM sodium orthovanadate, 2 mM PMSF, 2 mM NEM, 1 μg/ml pepstatin A) for 15 minutes on ice. Thereafter the lysates were clarified by centrifugation at 14,000 ***g*** for 15 minutes. The spun lysates were precleared with ProteinA-sepharose (Amersham Biosciences) for 1 hour at 4°C. The beads were removed by centrifugation, and the supernatant was divided in two and incubated for 3 hours at 4°C with protein A-sepharose beads precoupled to either anti-p130Cas or anti-paxillin antibodies. Lysates used for precipitation of paxillin were later used to immunoprecipitate FAK. The beads were washed twice with RIPA buffer and subjected to SDS-PAGE followed by wet transfer to a nitrocellulose membrane (Scheicher and Schnell). Tyrosine phosphorylated proteins were detected by anti-

	Cytoplasmic region
GD25β1A	HDRREFAKFEKEKMNAKWDTGE**NPIY**KSAV**TT**VV**NPKY**RKG
GD25β1AY783A	HDRREFAKFEKEKMNAKWDTGE**NPIA**KSAV**TT**VV**NPKY**RKG
GD25β1AY783F	HDRREFAKFEKEKMNAKWDTGE**NPIF**KSAV**TT**VV**NPKY**RKG
GD25β1AY795F	HDRREFAKFEKEKMNAKWDTGE**NPIY**KSAV**TT**VV**NPKF**RKG
GD25β1AYY783/795FF	HDRREFAKFEKEKMNAKWDTGE**NPIF**KSAV**TT**VV**NPKF**RKG
GD25β1AT788A	HDRREFAKFEKEKMNAKWDTGE**NPIY**KSAV**AT**VV**NPKY**RKG
GD25β1AT789A	HDRREFAKFEKEKMNAKWDTGE**NPIY**KSAV**TA**VV**NPKY**RKG
GD25β1ATT788-9AA	HDRREFAKFEKEKMNAKWDTGE**NPIY**KSAV**AA**VV**NPKY**RKG
GD25β1B	HDRREFAKFEKEKMNAKWDTVS YKTS KKQSGL

Fig. 3. β1 constructs transfected into GD25 cells. The conserved NPXY motifs and the threonine-rich region are indicated.

phosphotyrosine antibodies conjugated with horseradish peroxidase (RC20) and then enhanced chemiluminescence (Amersham Biosciences). To confirm that an equal amount of proteins was immunoprecipitated in all samples, the antibodies were stripped from the membrane by incubation in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, 100 μM β-mercaptoethanol for 30 minutes at 50°C, whereafter membranes were restained with relevant antibodies.

Immunofluorescence

Cover slips were coated with GST-invΔ1 (10 μg/ml) overnight at 4°C and blocked with 1% heat-treated BSA in PBS at 37°C for 1 hour. Cells were detached and incubated in serum-free DMEM containing cycloheximide (25 μg/ml; Sigma-Aldrich, Stockholm, Sweden) on BSA-blocked plates for 40 minutes at room temperature, then the GRGDS peptide (0.1 mg/ml) was added and the cells were incubated for an additional 20 minutes. After this, 5×10^4 cells were allowed to attach on invasin-coated cover slips at 37°C for 3 hours. Unattached cells were washed away and the remaining cells were fixed in 2% paraformaldehyde for 10 minutes. Alternatively, 2.5×10^4 cells were seeded on cover slips and cultured at 37°C for 20 hours in serum-containing medium. Subsequently the wells were washed and the cells were fixed as above. The fixed cells were permeabilized with 0.5% Triton X-100 and further processed for immunofluorescence labeling. To visualize β1-integrins, cells were incubated with primary antibodies against β1-integrins (HMβ1-1) and then with the FITC-conjugated goat anti-Armenian hamster secondary antibodies. In double labeling experiments, the cells were incubated as above followed by incubation with antibodies against phosphotyrosine (PY20) or vinculin and with the LRSC-conjugated secondary donkey anti-mouse antibodies. All incubations were performed at room temperature. Samples were mounted onto microscope slides using ProLong Antifade (Molecular Probes, Leiden, The Netherlands). The pictures were captured using a microscope (Zeiss axioskop 50) and a CCD camera (ORCA, Hamamatsu) and processed using Adobe software (Adobe).

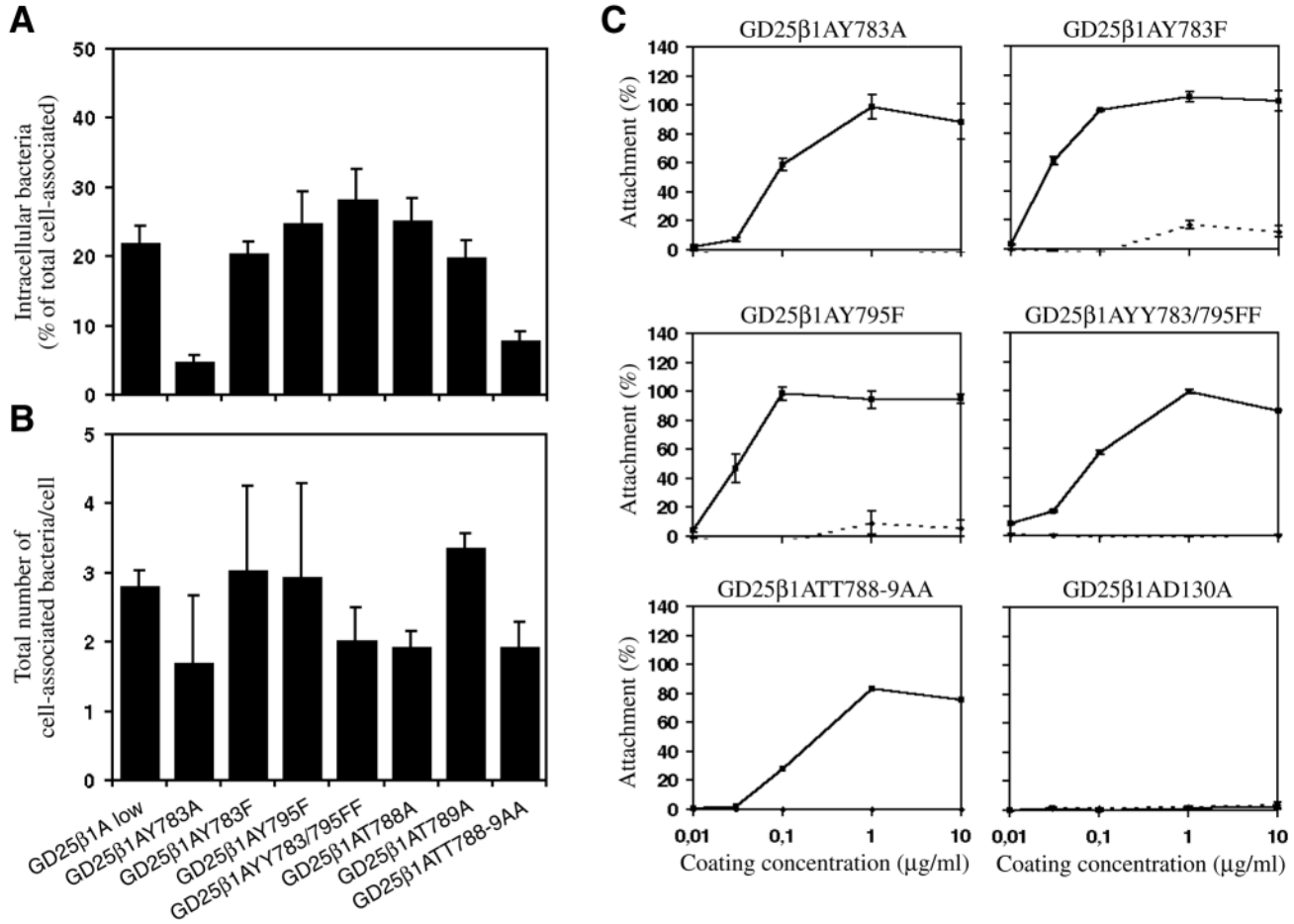


Fig. 4. Internalization of *Yersinia* and invasin-attachment by GD25 cells expressing different β1A-integrin mutants. Cells were infected with *Yersinia* at a calculated bacteria:cell ratio of 150:1 for 30 minutes at 37°C, and thereafter fixed and stained for extracellular and total cell-associated bacteria respectively. (A) The percentage of cell-associated bacteria located intracellularly is shown. (B) The total number of bacteria associated to one cell. The values represent the means±s.e.m. of at least five separate experiments. (C) The indicated cells were allowed to bind to microtiter plates coated with GST-invasin (solid line), GST alone (dotted line) or vitronectin for 1 hour. The number of cells adhering to vitronectin (coated at 10 μg/ml) represents 100% of attached cells.

Table 1. Level of β1-integrin expression determined by FACS analysis

Cell lines	FACS (mean)
GD25	8
GD25β1A high	62
GD25β1A low	30
GD25β1B	36
GD25β1AY783A	19
GD25β1AY783F	35
GD25β1AY795F	24
GD25β1AYY783/795FF	37
GD25β1AT788A	28
GD25β1AT789A	33
GD25β1ATT788-9AA	22

The values are means of the fluorescence peak channel from three separate experiments. The GD25 values are to be considered as background. The FACS analysis was performed as described in the Materials and Methods.

Results

Binding of β1-integrins to invasin is not sufficient for uptake of bacteria

To verify that the uptake process of bacteria to be studied was strictly dependent on β1-integrins, the cell lines GD25, GD25β1A and GD25β1B were exposed to the *Yersinia* MYM strain for 30 minutes, after which the level of binding and internalization of bacteria was determined. The *Yersinia* MYM strain is mutated in genes encoding Yop effectors and is internalized by both professional phagocytes and other normally non-phagocytic cells (Persson et al., 1997; Persson et al., 1999). The GD25 cells did not bind to *Yersinia* at all, whereas cells expressing β1A-integrins bound and internalized bacteria (Fig. 1A,B), thus, showing the specificity of this experimental setup. Cells expressing β1B-integrins bound to bacteria to the same extent as those expressing β1A-integrins, but they did not mediate internalization (Fig. 1A,B). GD25β1A low and GD25β1B expressed about similar levels of the β1-integrin, as shown by FACS analyses (Table 1).

To further evaluate the β1-integrin–invasin interaction, cells were plated for 1 hour onto dishes coated with invasin or vitronectin. With this setup, the ligand is immobilized on a surface and the integrins on the cells are forced to find its ligands, in contrast to the bacterial uptake assay where the bacteria are involved in finding receptors on the cells (Andersson et al., 1999). The recombinant invasin protein used was a truncated derivative corresponding to the 500 C-terminal amino acids of invasin, containing both the dimerization and integrin recognition parts (Dersch and Isberg, 1999). Vitronectin, which is a ligand for αvβ3-integrin but not for β1-integrins, was used to set the 100% attachment for each cell line. GD25 and GD25β1A cells attached to vitronectin to a similar extent, but, as in the uptake assay, only the GD25β1A cells bound to invasin (Fig. 1C). Cells expressing a β1A subunit unable to bind to ligand because of the mutated extracellular domain (GD25β1AD130A) (Takada et al., 1992) failed to attach to invasin. However, GD25β1B-expressing cells readily bound to invasin-coated surfaces, although somewhat less efficiently than the β1A-expressing cells (Fig. 1C). Hence, invasin can bind to β1-integrins exhibiting an inactive conformation for binding to fibronectin, but the binding itself is insufficient to promote integrin-mediated internalization.

Ligation and clustering of β1-integrins is associated with tyrosine phosphorylation of several focal adhesion proteins (Vuori, 1998). The β1B isoform does not mediate such phosphorylations and neither does it promote formation of focal adhesion structures when exposed to ECM proteins (Balzac et al., 1993; Balzac et al., 1994; Armulik et al., 2000). This is believed to be because of the lack of ligand binding, and

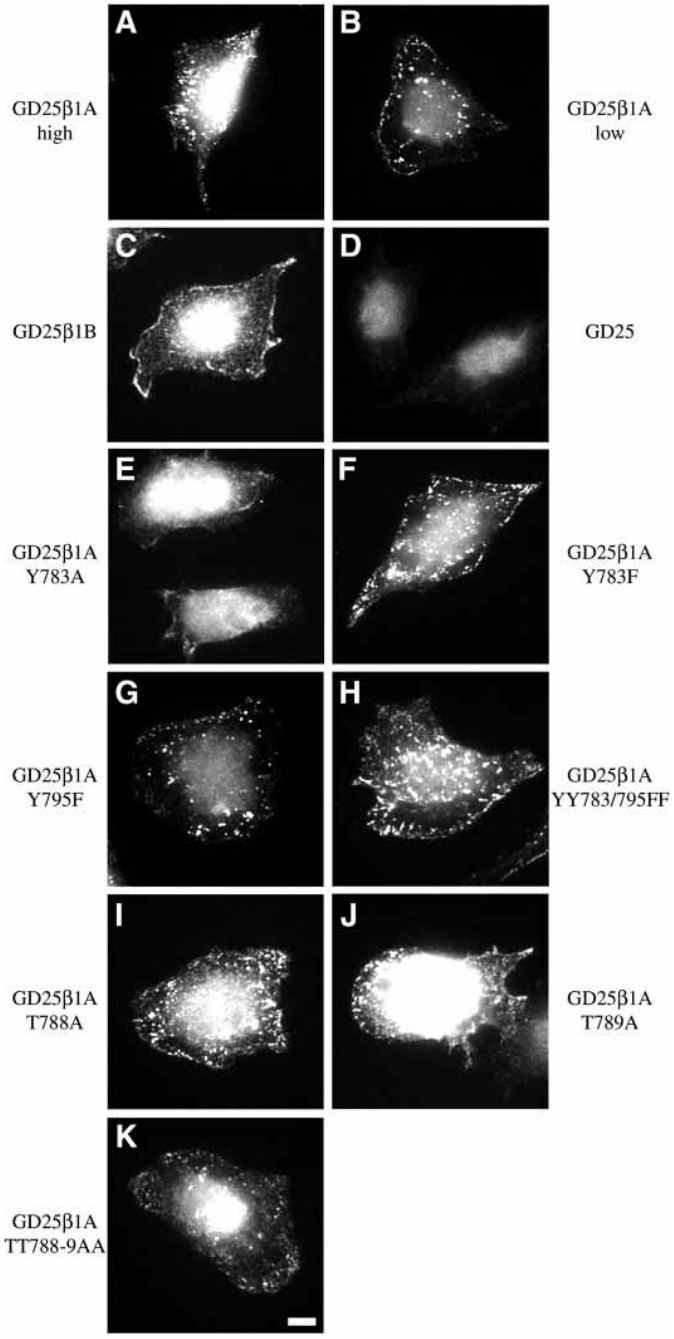
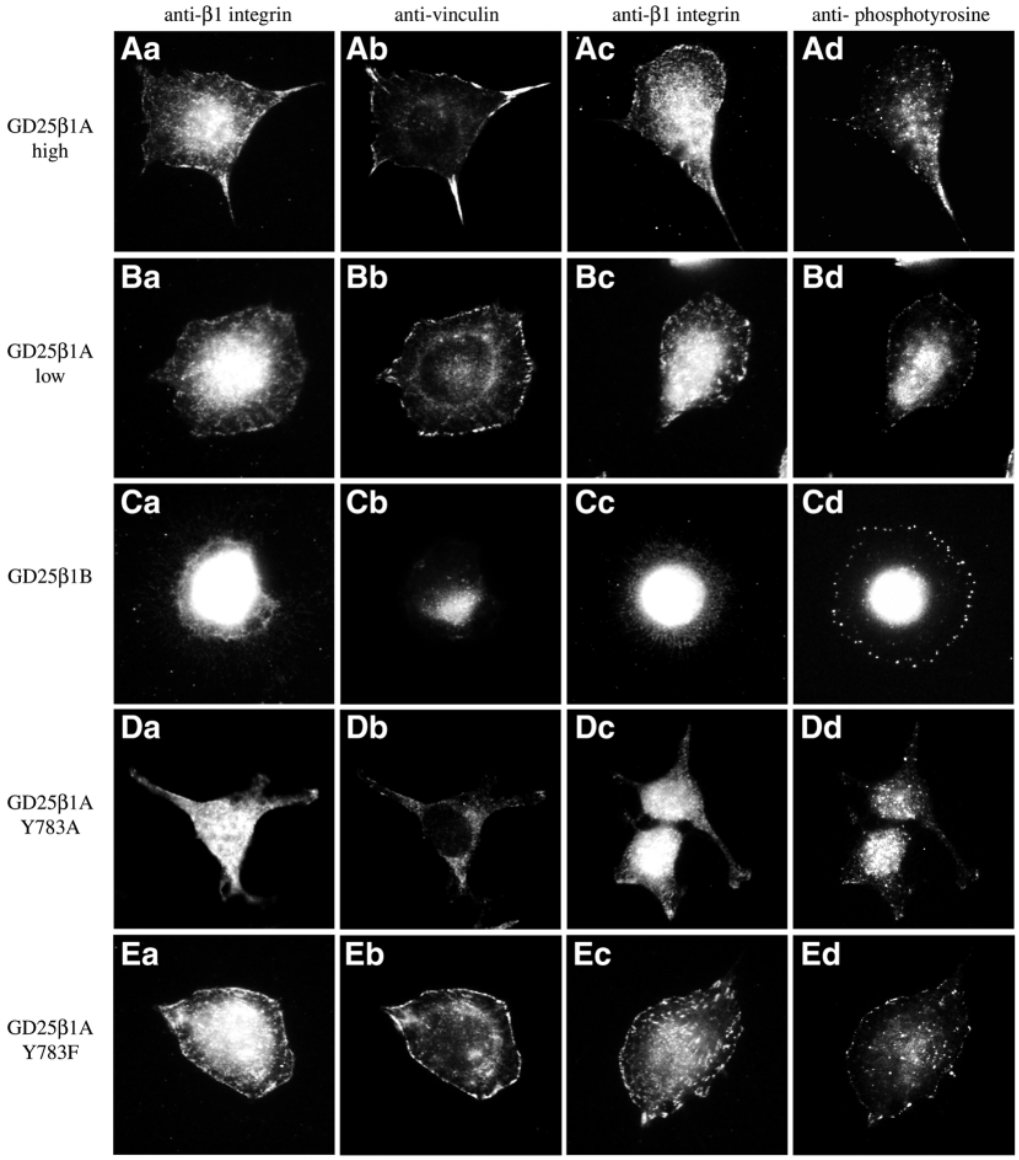


Fig. 5. Distribution of the different β1-integrin variants in cells grown in culture. The indicated cells were seeded on glass cover slips and incubated overnight in culture medium after which they were fixed and stained by anti-β1-integrin antibodies followed by fluorescein-conjugated secondary antibodies. The specimens were analyzed using a fluorescence microscope, and images were taken using a CCD camera. Bar, 10 μm.



therefore it was of interest to investigate invasin-induced β1B-integrin signaling. To elucidate this, GD25 cells expressing the β1A or the β1B subunit were plated on invasin-coated dishes, and the focal contact components FAK, p130Cas and paxillin were immunoprecipitated from cell lysates. Western blotting using anti-phosphotyrosine antibodies showed that all three proteins were tyrosine phosphorylated in an invasin-dependent manner in cells expressing β1A (Fig. 2). By contrast, in β1B-expressing cells no induced tyrosine phosphorylation of FAK or paxillin and only a slight increase in p130Cas phosphorylation was seen after adhesion to invasin. Hence, the occupancy of the β1B-integrins is not sufficient for induction of tyrosine phosphorylation of focal contact components.

Phosphorylation of the β1-integrin NPXY or TT motifs are not important for bacterial uptake

The inability of GD25 cells expressing the β1B subunit to internalize *Yersinia* indicated that the cytoplasmic region specific to β1A was required for bacterial uptake. There are several potential phosphorylation sites in this region, among these two tyrosines (amino acid 783 and 795 in murine β1A), which are within the NPXY motifs, and two threonines (amino acids 788 and 789 in murine β1A) located between the two NPXY motifs. These sites have been implicated in downstream signaling activities, such as FAK activation (Wennerberg et al., 1998; Wennerberg et al., 2000) and were therefore of particular interest. To elucidate the importance of these motifs for bacterial internalization, uptake studies were performed using GD25 cells expressing β1A mutants with the tyrosines exchanged to phenylalanines (GD25β1AY783F, GD25β1AY795F and GD25β1AYY783/795FF; Fig. 3) or to alanine (GD25β1AY783A), or with the threonines exchanged to alanines (GD25β1AT788A, GD25β1AT789A and GD25β1ATT788-9AA). The β1AY783A- or β1ATT788-9AA-expressing cells showed a marked reduction in bacterial internalization, whereas cells expressing the tyrosine to phenylalanine mutants or the single threonine mutants were as effective as wild-type β1A (Fig. 4A). Hence, these results indicate that phosphorylation of these motifs is dispensable for

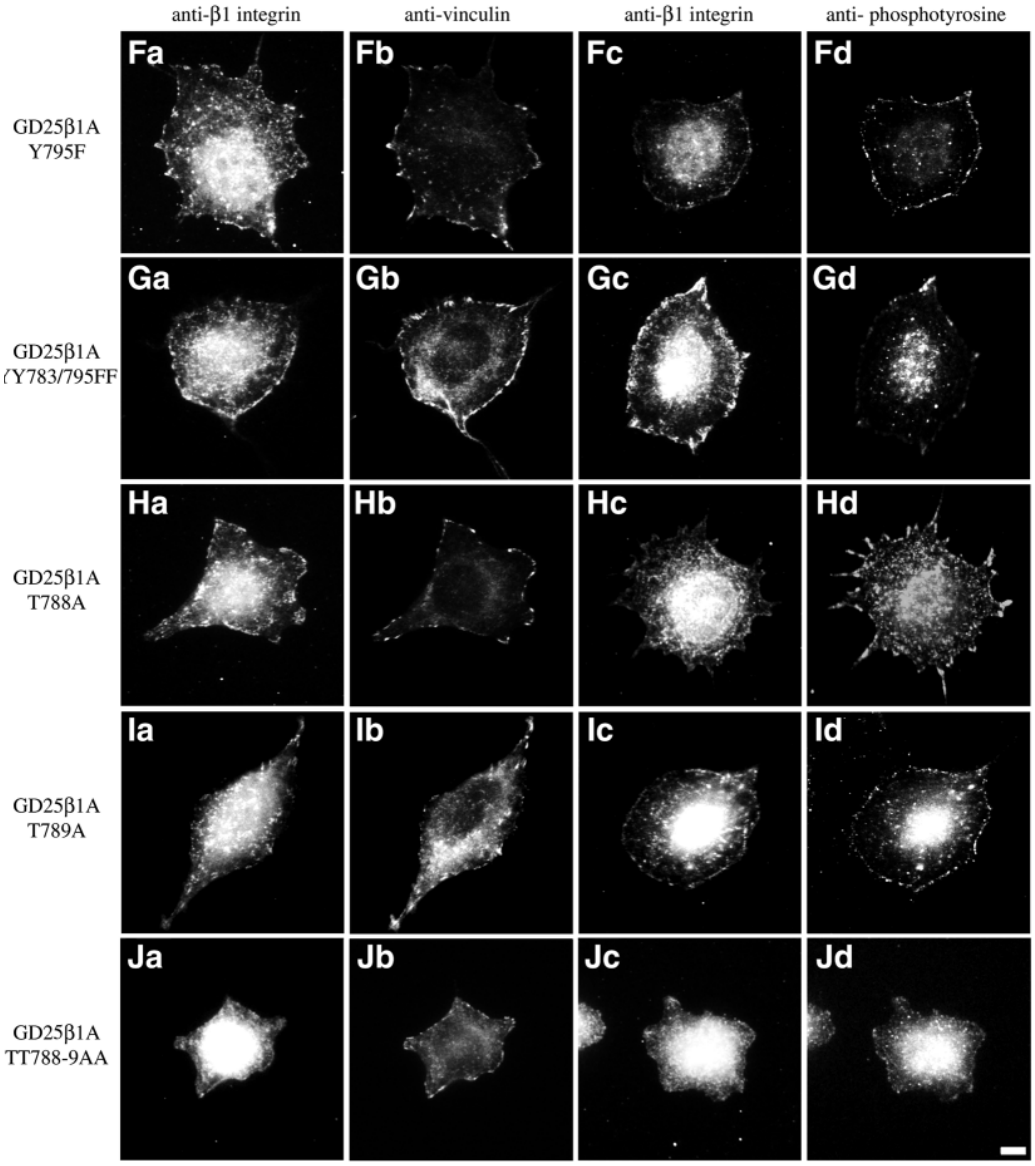


Fig. 6. Distribution of the different β1-integrin variants in cells adhering to invasin. GD25 cells expressing different variants of the β1-integrin were allowed to spread on GST-invasin-coated glass cover slips for 3 hours. The cells suspended in serum-free DMEM were pretreated with cycloheximide (25 μg/ml) and GRGDS (0.1 mg/ml). After fixation, the cells were double-stained with anti-β1-integrin (Aa-Ka) and anti-vinculin (Ab-Kb) antibodies or with anti-β1-integrin (Ac-Kc) and anti-phosphotyrosine (Ad-Kd) antibodies. The specimens were analyzed using a fluorescence microscope and images were taken using a CCD camera. Bar, 10 μm.

internalization. GD25β1AY783F, GD25β1AY795F and GD25T789A cells bound to as many bacteria as cells expressing wild-type β1A, whereas those expressing β1AY783A, β1ATT788-9AA, β1AYY783/795FF and β1AT788A bound to slightly less bacteria (Fig. 4B). However, the uptake efficiencies by the two latter were similar or higher compared to cells expressing wild-type β1A, indicating that the amount of bound bacteria is not correlated with internalization efficiency. Moreover, there was no striking difference in integrin-expression (Table 1) or in adhesion to invasin-coated surfaces between the different β1A-integrin mutants (Fig. 4C).

Cells affected in bacterial uptake also have altered spreading

Earlier work has suggested that peripheral focal-complex-like structures play a role in uptake of *Yersinia* (Persson et al., 1997; Persson et al., 1999). Therefore it was of interest to investigate whether the different β1-integrin mutants were affected in their ability to form focal contact structures. For this analysis, cells expressing different mutants were seeded on cover slips and incubated overnight in similar conditions to those used in uptake studies. The cells were then fixed and the integrins were visualized by indirect immunofluorescence using β1-integrin-specific antibodies. All cells, including GD25, exhibited spread morphology, which was expected since they all express integrin αVβ3, which will bind to serum-derived vitronectin. β1-integrins were detected in all cells (apart from GD25), and they localized to focal-contact-like structures except in cells expressing β1B or β1AY783A where the β1-integrins were distributed along the cell periphery (Fig. 5).

To evaluate invasin-induced focal contact formation, the cells were allowed to adhere to invasin for 3 hours under serum-free conditions. After this, the cells were fixed and double stained with β1-integrin-specific antibodies together with antibodies against the focal contact marker vinculin or with antibodies against phosphotyrosine. To exclude binding of αVβ3 to secreted matrix proteins, the cells were pretreated with

cycloheximide; in addition, the GRGDS peptide was included at a concentration known to block αVβ3 binding without severely affecting β1A-mediated attachment to fibronectin (Wennerberg et al., 1998). With this pretreatment, the GD25 cells did not attach to invasin-coated dishes (data not shown). The β1A-expressing cells spread with a uniform morphology, in some cases with a pointed end, and they formed vinculin-, phosphotyrosine- and β1-integrin-containing focal contacts at the edges as well as central focal adhesion-like structures spread throughout the cell (Fig. 6A,B). The cells expressing the single and double tyrosine to phenylalanine β1A mutants spread and formed focal contacts similar to cells expressing wild-type β1A (Fig. 6E-G). The β1B-expressing cells did adhere to the invasin surface, but were clearly affected in spreading. No focal contact structures containing vinculin could be observed at cell edges, only a diffuse staining surrounding the nucleus could be seen (Fig. 6Ca-c). Noteworthy, despite the lack of detectable focal adhesion markers, are the very weak β1-integrin staining and clear punctuate structures of phosphotyrosine staining observed mainly at the very edges of the cells (Fig. 6Cd). The GD25β1AY783A cells, which like GD25β1B cells exhibited a reduced ability to internalize *Yersinia*, were also affected in spreading on invasin. These cells spread in a 'neuron-like' pattern with long protrusions extending from the center of the cells (Fig. 6D). The β1-integrins as well as vinculin were distributed all over the cells with some accumulation at the tip of the extensions (Fig. 6D). Here, a somewhat different pattern was also seen for phosphotyrosine proteins, which could be seen in punctuate structures, although they were not as distinct as in GD25β1B cells (Fig. 6Dd). The double threonine mutant of β1A that also was affected in bacterial uptake was markedly delayed in spreading compared with wild-type β1A, although focal-complex-like structures could be seen at the cell edges (Fig. 6J). The single threonine to alanine mutants of β1A resembled wild-type β1A in spreading and focal contact localization of integrin, vinculin and phosphotyrosine (Fig. 6H,I).

Discussion

In this paper we show that binding of invasin-expressing bacteria to β1-integrins on cells is insufficient for induction of internalization of the bacteria. We also show that mutations causing severe alterations in the integrin cytoplasmic region containing the membrane proximal NPIY motif and the neighboring double threonine residues result in reduced uptake of bacteria. In addition, we demonstrate a correlation between bacterial internalization and invasin-stimulated spreading, where transfectants that are unable to form peripheral adhesion structures are also defective in bacterial internalization. The experimental setup used in this study with invasin and GD25 cells transfected with different variants of the β1 subunit was shown to provide an optimal tool for investigating integrin signaling. The GD25 cells allow studies of β1-integrin without interference from endogenous counterparts, and invasin, in contrast to for example fibronectin, binds exclusively to β1-integrins.

The ability of β1B-integrins as well as the β1ATT788-9AA to bind to invasin-expressing bacteria and attach to invasin-coated surfaces was somewhat surprising since these variants have been found to be defective in binding to physiological ligands (e.g. fibronectin and laminin) (Retta et al., 1998; Wennerberg et al., 1998; Armulik et al., 2000). There was, however, a discrepancy between the binding of bacteria and adhesion to invasin-coated surfaces, where the β1B-expressing cells showed lower binding compared with the β1A-expressing cells in the latter case. Thus, the binding of invasin to β1B is obviously weaker than that to β1A, but still sufficient for binding bacteria. It is likely that the bacteria present a denser and defined surface of correctly exposed ligands compared with that on the coated dish where the binding epitope is likely to be distributed more randomly. In addition, the shear force during the washing procedure is higher in the cell attachment assay than in the bacterial binding assay.

β1B-expressing cells binding to invasin had an impaired signal transduction as tested by immunoprecipitation experiments. Furthermore, they showed a defective spreading on invasin. It is obvious that this integrin isoform does not participate in establishing typical focal adhesion sites on the invasin substrate, in analogy with previous studies of fibronectin and laminin (Balzac et al., 1993; Armulik et al., 2000). However, our finding of the involvement of the so far non-identified phosphotyrosine protein complex(es) in spreading GD25β1B cells implies that some kind of signaling is associated with this truncated receptor. The signaling can be derived from either the very short β1B cytoplasmic tail or from the associated α-chain. Interestingly, p130Cas was weakly tyrosine phosphorylated in response to adhesion to invasin, which suggests that p130Cas could be activated upon β1B binding to invasin. Work to identify the phosphotyrosine protein(s) is currently ongoing, and this will hopefully contribute to a better understanding of the molecular events involved in formation of integrin adhesions, dissecting early microspike-associated signaling from that associated with later events (lamellipodia and final adhesion).

The ability of β1B-integrins to bind to invasin without being able to internalize invasin-expressing bacteria clearly indicates that bacterial internalization is an active process and that the cytoplasmic tail of the β1A subunit mediates the necessary signaling. However, when analyzing the importance of different amino acid motifs in the β1A-integrin cytoplasmic tail, only mutations causing more severe alterations in the structure resulted in reduced internalization capacity. The finding that the Y783F mutation is tolerated, whereas the Y783A mutation abrogates internalization of bacteria, implies that the NPIY motif is important for uptake but that there is no need for tyrosine phosphorylation of the NPXY motifs for bacterial internalization to occur. This result is consistent with the fact that the corresponding motif in the major phagocytic receptor, integrin αMβ2 (CD11b/CD18, Mac-1), that is present on professional phagocytes contains phenylalanine instead of tyrosine at this position (Kishimoto et al., 1987; Law et al., 1987). Moreover, a tyrosine to glutamic acid mutation in the first NPXY motif of β1-integrins has been shown to result in an integrin that is unable to internalize bacteria (Tran Van Nhieu et al., 1996). Since glutamic acid may mimic a phosphorylated tyrosine (Maciejewski et al., 1995), this finding suggests that phosphorylation of this residue is unfavorable for internalization. However, the more drastic mutation of the tyrosine in the first NPXY motif to alanine probably disturbs the predicted tight turn conformation at this motif (Haas and Plow, 1997), and this could explain the

inability for this mutant to mediate internalization of bacteria. Similarly, different effects on integrin localization to focal contact structures when exchanging the tyrosine in the integrin NPIY motif to phenylalanine or to alanine had been seen for chicken β1-integrins overexpressed in a background of wild-type β1-integrins in NIH3T3 cells (Reszka et al., 1992). Under these conditions the phenylalanine variant localized to focal contacts upon adhesion to fibronectin, whereas the corresponding alanine variant exhibited reduced localization to these sites (Hayashi et al., 1990; Reszka et al., 1992). The defective internalization of bound bacteria by the β1ATT788-9AA mutant integrin could be caused by the removal of phosphorylation sites or by conformational disruption. Since mutations of the individual threonines did not have any major effects, the first alternative appears unlikely. These threonines are flanked by hydrophobic amino acids, and the change of both threonines to alanines will remove the intervening polar groups and markedly change the nature of the region between the two NPXY motifs. The importance of keeping these regions structurally intact probably reflects that they are part of interaction domains important for downstream signaling. Both the NPXY and the double threonines have been implicated as interaction domains for cytoskeletal and signaling proteins (Burridge and Chrzanowska-Wodnicka, 1996; Liu et al., 2000). The Y783A mutation of β1A disrupts the binding of the cytoskeletal proteins talin and filamin to the integrin (Pfaff et al., 1998; Kääpä et al., 1999), and the TT788-9AA mutation impairs binding of the integrin interactive protein ICAP-1 (Stroeken et al., 2000; Degani et al., 2002). However, since the β1ATT788-9AA, like β1B-integrins, also has an altered extracellular conformation (Wennerberg et al., 1998), it can not be excluded that part of the observed effect on bacterial internalization capacity is caused by a lower affinity for invasin.

A common feature for the mutations causing reduced capacity to internalize *Yersinia* was that they also affected cell spreading on immobilized invasin. This correlation is not surprising since both processes involve integrin-mediated adhesion and membrane extensions. The *Yersinia* virulence factor YopH, which mediates phagocytic inhibition of target cells, specifically disrupts peripheral focal contact structures, and YopH that are deficient in focal adhesion recognition exhibit a reduced inhibitory effect (Persson et al., 1997; Persson et al., 1999). Peripheral focal contact structures have therefore been implicated as important for uptake of *Yersinia*. In accordance, all β1-integrin variants that could mediate uptake of *Yersinia* also localized to peripheral focal contact structures, albeit that these structures were not always morphologically similar to wild-type focal contacts, and two out of three mutations that affected uptake also caused a dislocation of the integrin from peripheral focal contacts. This suggests that the capacity to form peripheral focal complex structures might be important, but not sufficient, for integrin-dependent bacterial internalization. Tran Van Nhieu et al. have reported that several integrin mutants that exhibited reduced focal contact association were more efficient in promoting bacterial uptake (Tran Van Nhieu et al., 1996). This might appear contradictory to our data, but since that study was made with a background of endogenous wild-type β1-integrins it is difficult to draw firm conclusions on the effect of the mutations. However, in spite of the different systems used, it is noteworthy that in both cases several of the integrin mutants exhibiting reduced focal contact localization were still observed in peripheral complex-like structures. Furthermore, FAK null cells, which have more stable and higher numbers of focal contacts compared with cells expressing FAK (Ilic et al., 1995), exhibit an impaired capacity to internalize bacteria (Alrutz and Isberg, 1998) in addition to being defective in cell migration. Since FAK is implicated in the turnover of focal contacts, these data indicate that it is the dynamics of focal complexes rather than the presence of such structures per se that is important in the bacterial uptake process. This appears likely, considering that bacterial internalization is a dynamic process involving initial adhesion of bacteria to the cell surface followed by membrane extensions and closure of a phagosome. Hence, if the ability to form and reshape focal contact-like structures is critical for bacterial internalization, integrin variants that are less potent in forming rigid and stable focal adhesions may be more efficient, whereas variants that cannot form these structures at all, in this case β1B and β1AY783A, are defective in phagocytosis. In the case of the double threonine to alanine mutant the receptor was defective in uptake and spreading, even though it could localize to peripheral focal-contact-like structures. It is possible that the nature of these adhesion complexes differ from these of uptake-competent integrins and that there are certain protein interactions that are required at some step of the internalization process and which fail because of the missing threonines.

This work was supported by grants from the Swedish Medical Research Council, the Swedish Cancer Foundation, the Infection and Vaccinology Research Program of the Swedish Foundation for Strategic Research, the King Gustavs Vth 80 year Foundation and Medical Faculty Research Foundation at Umeå University.

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