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INTERNALIZATION OF THE $\alpha_5\beta_1$ INTEGRIN DOES NOT DEPEND ON "NPXY" SIGNALS

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The $\alpha_5\beta_1$ integrin is a constitutively internalized fibronectin receptor. It contains in the cytoplasmic tail of its β_1 subunit two NPXY sequences which have been proposed to mediate internalization. Indeed a NPXY motif constitutes the internalization signal for the Low Density Lipoprotein (LDL) and insulin receptors. To learn more about the putative role of the two NPXY sequences in internalization of the $\alpha_5\beta_1$ receptor, we have made and expressed mutants of the human β_1 subunit in Chinese Hamster Ovary (CHO) cells, in which the two tyrosines of the NPXY motifs were replaced by serine residues. A cytoplasmic variant β_{1B} which does not contain any NPXY sequence was also analyzed. Our results indicate that the NPXY mutants and the cytoplasmic variant are still internalized. Thus in the $\alpha_5\beta_1$ receptor, the highly conserved NPXY sequences do not function as internalization motifs.

The integrins are a large family of heterodimeric ($\alpha\beta$) transmembrane receptors that mediate cell-cell and cell-extracellular matrix (ECM) interactions (1, 2). Cell-substratum adhesion occurs at sites of the plasma membrane called focal contacts or adhesion plaques, where some integrins are concentrated. They provide a mechanical transmembrane linkage between the ECM and the cytoskeleton (3-5). At these sites, cytoskeletal proteins such as talin and α-actinin are thought to interact directly with cytoplasmic domain of the β_1 subunit of integrins (6, 7). In addition to this structural role, integrins also function as transduction receptors for two types of signals: "outside-in" signals from the matrix to the cell promoting a variety of cellular responses (8-10) and "inside-out" signals from cytoplasm that affect the adhesive properties of integrins (11). Among the integrins, the short cytoplasmic domain of the β_1 subunit has focused attention on its role in signal transduction and its interaction with cytoskeletal components. Several experiments of deletions and point mutations of this 47 amino acid cytoplasmic domain demonstrated that it contains the information targeting integrins to adhesion plaques (12-14). Recently Reszka *et al.* (15) have pointed to three putative regions as involved in the localization to focal contacts.

In addition to its well-studied role as a link with cytoskeletal components, the β_1 cytoplasmic domain contains two NPXY sequences, which are supposed to allow the

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internalization of integrins. Indeed the $\alpha_5\beta_1$ fibronectin receptor has been shown to be constitutively internalized in CHO cells in suspension (16, 17), or in fibronectin-adherent CHO cells (18). The NPXY sequence is the motif responsible for the internalization of the LDL and insulin receptors (19, 20). This latter sequence, found in the cytoplasmic domain of these two recycling receptors, adopts a tight turn conformation and it is supposed to interact with adaptor proteins in clathrin-coated pits (21, 22). In several other recycling receptors common stuctural features and chemistry of internalization sequences have been defined, and an exposed tight turn has been implicated as the general conformational recognition motif for endocytosis (23, 24).

Herein, we address the question of a possible involvement of the two NPXY putative internalization motifs found in the cytoplasmic domain of the β_1 chain during the endocytosis of the $\alpha_5\beta_1$ fibronectin receptor. We have made three mutants of the human β_1 chain expressed in CHO cells, in which the two tyrosines of the NPXY motifs were replaced by serine residues, either individually or in combination. It has been shown that this substitution gave an internalization-negative sequence for the LDL receptor (21). Our results demonstrated that the replacement of the NPXY motifs by NPXS did not impair the internalization of the receptors. These findings were further supported by the observation that the cytoplasmic variant β_{1B} (25, 26) which does not contain any NPXY sequence, was still able of recycling when expressed in CHO cells. Thus in the $\alpha_5\beta_1$ receptor, the highly conserved NPXY sequences do not function as internalization motifs.

EXPERIMENTAL PROCEDURES

Cell culture.
Wild-type CHO cells were grown on plates at 37°C in a humidified 5% CO_2/95% air atmosphere in Minimum Essential Medium with alpha modification (α MEM) without nucleoside and supplemented with 7,5% fetal calf serum (v:v). Cells were harversted with Phosphate Buffer Saline (PBS) solution supplemented with 1 mM EDTA and 0,05% trypsin (w:v).

Site-directed mutagenesis of the human β_1 cDNA and expression of mutant subunits in CHO cells.
The full-length cDNA clone of the human β_1 subunit was generously provided by Dr Ruoslahti (27). The β_1 cDNA was cloned into the pALTER vector and the three mutants SY,YS, and SS β_1 cDNAs were generated using an in vitro mutagenesis kit from Promega (Altered Sites in vitro mutagenesis system). Each mutant was screened by sequencing the β_1 cDNA (Sequenase vers. 2.0; USB). CHO cells (2.10^6 cells) were transfected for 4 hours with 5 µg of SV-40-based expression vector pECE (28) including the human wild type or mutant β_1 cDNAs and 0,5 µg pSV2-neo plasmid (29), using 15 µl Transfectam (Sepracore, France) in α MEM. After 48h the cells were selected for resistance to 400 µg/ml geneticin (Gibco), and 12 days later resistant colonies were collected by scraping with micropipette tips. The cells were sorted for high expression by immunofluorescent staining using the K20 mAb (Immunotech) directed against the human β_1 subunit. Clonal cell lines were obtained by plating at limiting dilution populations that expressed high level of human β_1.

Flow cytometry.
The cells were harvested, pelleted at 4°C, resuspended and fixed in PBS supplemented with 3% paraformaldehyde for 10 min at 37°C. The fixed cells were washed in Hank's Balanced Salt Solution (HBSS) and incubated with the anti-human β_1 subunit K20 mAb, or with a control mouse IgG for 30 min at 4°C. After two more washes, the cells were incubated for 30 min with fluorescein isothiocyanate (FITC)-coupled $F(ab')_2$ goat anti-mouse IgG (Jackson). Then 10000 cells were analyzed on a FACScan (Beckton Dickinson).

Immunofluorescent based internalization assay and confocal laser scanning microscopy.
To follow the internalization of β_1 mutant subunits, the K20 mAb which recognizes an extracellular epitope was used. With cells in suspension it is possible to obtain clear intracellular optical sections with a confocal laser scanning microscope. Harvested cells were incubated for 45 min at 4°C with the primary K20 mAb to stain the exogenous human β_1 subunit expressed at the surface of the plasma membrane. Then, they were incubated for 30 min at 37°C in α MEM/10 mM Hepes pH 7.8 to allow the internalization of the integrins and the receptor associated antibodies. Controls were kept at 4°C. Subsequently cells were fixed in 3% paraformaldehyde and then plated onto slides using a cytospin. Cells were then permeabilized by 0,3% Triton X 100 for 10 min, and a final incubation was performed for 30 min with Rhodamine-conjugated $F(ab')_2$ goat anti-mouse IgG (Jackson). The immunofluorescent staining was observed with a Zeiss confocal laser scanning microscope.

RESULTS

In order to test the possible involvement of the two NPXY sequences in the internalization process of β_1 containing integrins, three mutants of the human β_1 cytoplasmic domain in which the two NPXY motifs were disrupted were constructed. In two of them named SY and YS, a point mutation was performed: the tyrosine either in position 783 or 795 was replaced by a serine, respectively. The third mutant named SS was constructed with the substitution of both tyrosyl residues (Fig.1). All these mutations in the cytoplasmic domain of human β_1 integrin cDNA were performed *in vitro* by site-directed mutagenesis. This amino acid substitution was chosen because the substitution of tyrosine by serine in the NPXY motif of the LDL receptor was shown to lead to an internalization-negative sequence. Furthermore non aromatic residue replacement of the tyrosine is predicted to destabilize β-turn formation, whereas aromatic residue substitution has no effect on internalization and retains the β-turn (21).

The cDNAs encoding the normal β_1 and these mutant subunits were transfected into CHO cells. Several clonal cell lines expressing either the wild-type or a mutant β_1 subunit were selected by immunofluorescence using the K20 mAb recognizing specifically the human β_1 subunit. One representative from each type of clone was then examined in detail. A CHO cell

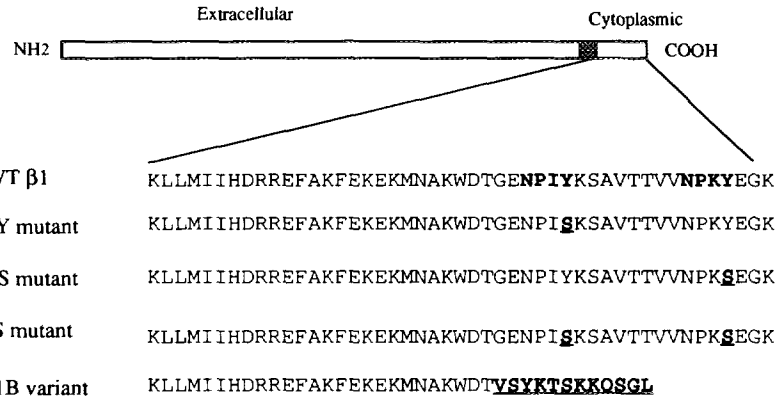


Fig.1. Cytoplasmic domain sequences of the β_{1B} variant subunit and β_1 mutant subunits.

line transfected with the cytoplasmic variant β_{1B} (26) was also analyzed in addition to the Tyr mutants. This β_{1B} subunit does not contain the NPXY sequences in its cytoplasmic tail (Fig.1), and was used as a control.

Since the K20 mAb recognizes an extracellular epitope of the human β_1 subunit, it allowed us to estimate by flow cytometry the surface expression levels of the different exogenous β_1 subunits: wild-type, variant, and mutants. Figure 2 shows that these constructs were expressed at the cell surface. Therefore, it is likely that the human β_1 chain was associated with a hamster α counterpart at the cell surface. In fact, it has been reported that in transfected CHO cells, the human β_1 subunit associates with endogenous hamster α_5 subunit to form a functional hybrid receptor to fibronectin that is correctly exported to the cell surface (30). A similar $\alpha_5\beta_1$ hybrid receptor was also formed with the chicken β_1 chain expressed in mouse cells (12, 13).

The internalization of these chimeric hamster α / human β_1 receptors was detected in an immunofluorescent based internalization assay. Cells expressing wild-type, variant, or mutant human β_1 were grown in suspension. The K20 mAb was bound to the cell surface human β_1 subunit after an incubation at 4°C, a temperature at which no endocytosis is expected to occur. Then the cells were warmed at 37°C for 30 min. to allow the internalization of surface receptors. Alternatively, control experiments were carried out at 4°C for 30 min. The cells were subsequently fixed by paraformaldehyde, permeabilized with Triton X100 and incubated with rhodamine-coupled $(Fab')_2$ goat anti-mouse IgG, to determine the cellular localization of the human β_1-K20 mAb complexes. With cells in suspension, we obtained well-defined intracellular optical sections, using a confocal laser scanning microscope. Figure 3 shows that the human β_1 molecules tagged with K20 can be detected at the cell surface and in vesicular structures observed in intracellular optical sections of the cells expressing the wild-type β_1 subunit, but also in cells expressing either SY, YS, or SS β_1 mutant. The three mutants exhibited staining patterns that were indistinguishable from wild type. In all these cells the K20-positive intracellular vesicles were only observed when an internalization step was performed at 37°C prior staining. Conversely, the omission of the high temperature incubation step in our assay resulted in a strong staining at the cell surface, but no intracellular vesicles containing K20 were detected. Indeed, the internalization of the K20 mAb was dependent on the internalization of the human β_1 chain since intracellular vesicles containing this antibody were never detected in non transfected CHO cells, with or without the incubation step at 37°C. Therefore the endocytosis of K20 reflected a true internalization of the β_1 subunit.

In a similar way, we found that the cytoplasmic variant β_{1B} which does not contain any NPXY sequence was also internalized (Fig.4). This latter result was consistent with the data obtained with the SY, YS and SS mutants. A similar internalization of K20-coupled human β_1 subunits was observed when the assay was performed on fibronectin-adherent cells (data not shown).

Taken together our results indicate that the disruption of the highly conserved NPXY motifs in β_1 containing integrins does not impair the internalization of these receptors.

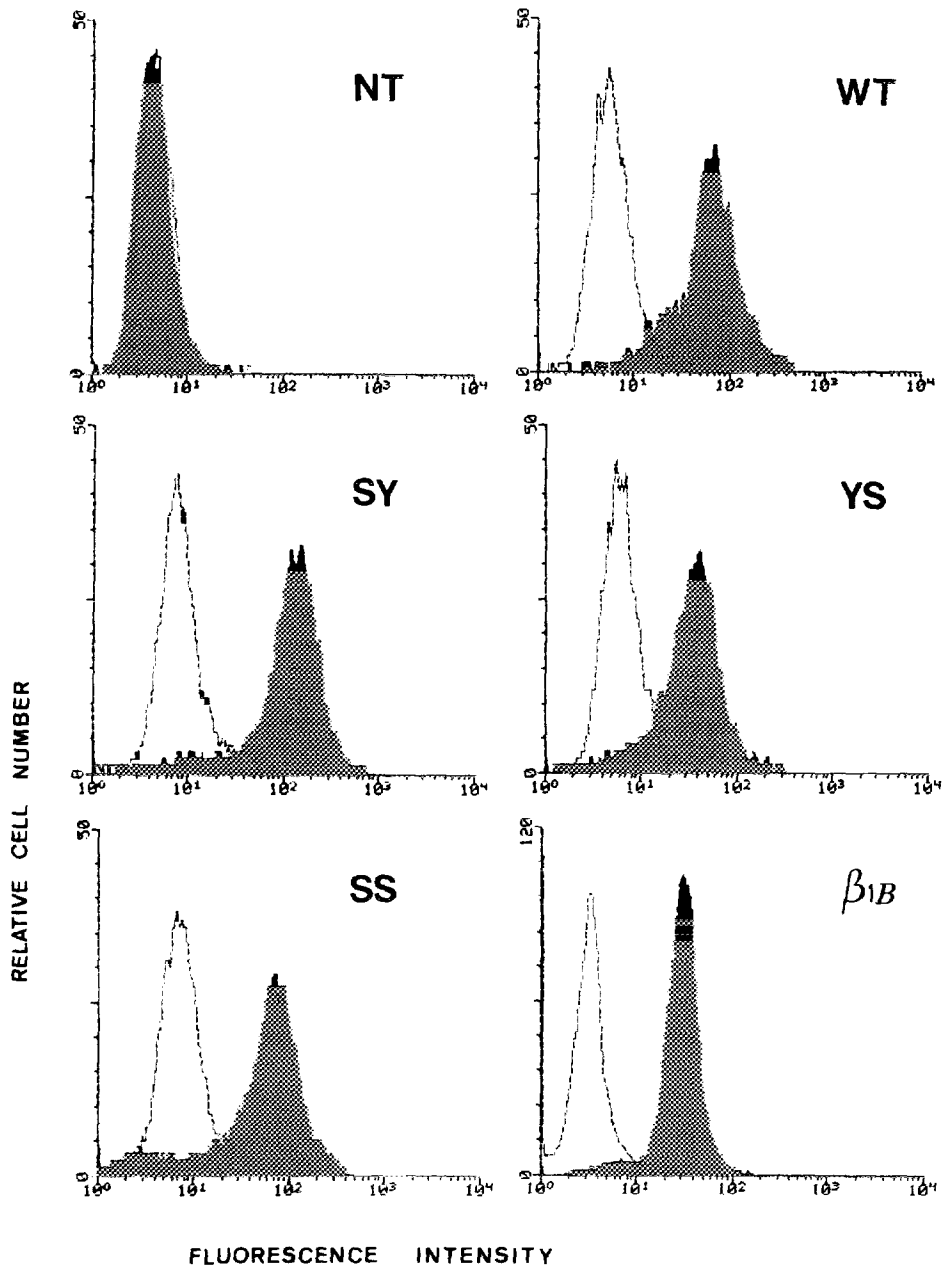
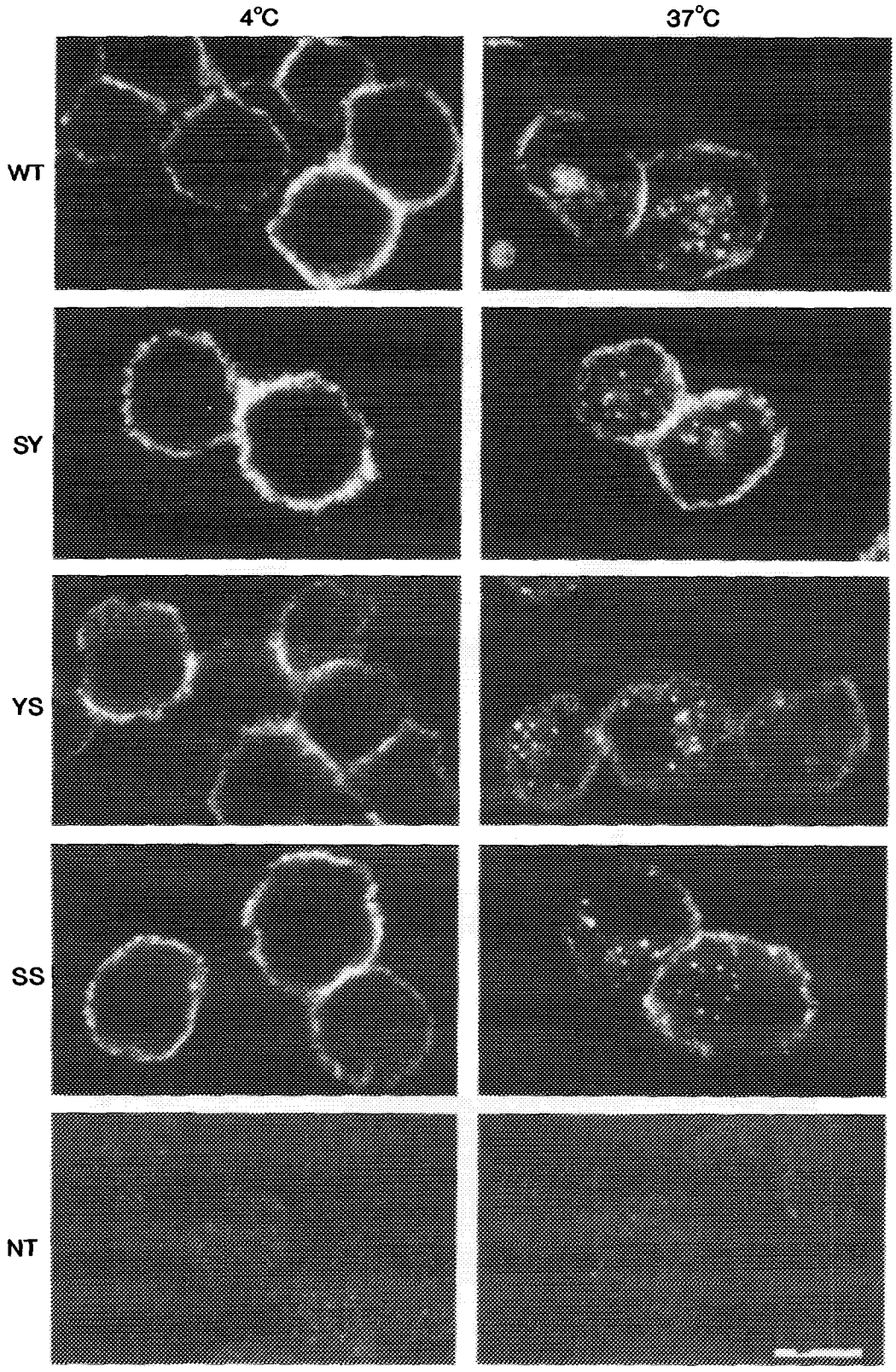


Fig.2. Cell surface expression of the human β1 subunit. Cells were fixed with 3% paraformaldehyde and incubated with a control antibody (left histogram) or the anti-human β_1 monoclonal antibody K20 (right histogram). After washes, the cells were incubated with a (FITC)-conjugated F(ab)'$_2$ goat anti-mouse IgG as described under Experimental Procedures. For each sample, the fluorescence intensity of 10,000 cells was measured by flow cytometry.

DISCUSSION

Previous studies have established that there are internalization signals in the cytoplasmic domains of constitutively recycled receptors that are believed to interact with the adaptor


4°C
37°C
WT
SY
YS
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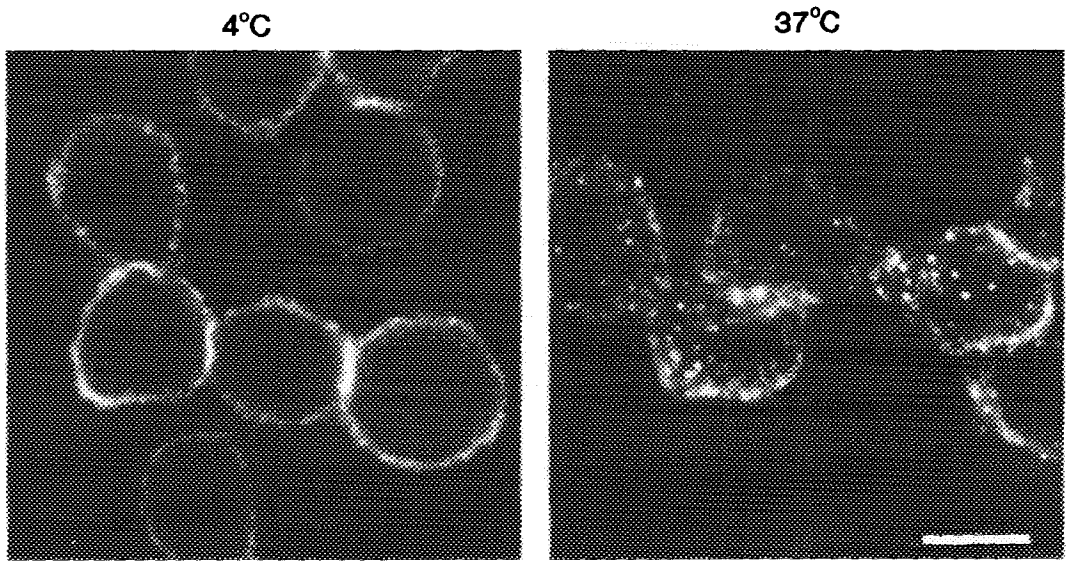

Fig.4. Internalization of a human cytoplasmic β_{1B} variant observed by confocal laser scanning microscope after immunofluorescent staining of suspension cells. Harvested cells in suspension were cell surface stained for the β_{1B} subunit with the primary K20 mAb anti-human β_1 at 4°C. Then the cells were incubated for 30 min at 37°C to allow internalization, or at 4°C for the controls. The cells were subsequentely fixed, plated onto slides using a cytospin, and permeabilized before incubation with Rhodamine-coupled F(ab)'$_2$ goat anti-mouse IgG. The optical sections into the stained cells (0.6 µm thick) were performed with a Zeiss confocal laser scanning microscope. Bar = 10 µm.

proteins of the coated pits. These internalization signals are self-determined three-dimensional structures, and a tight turn is implicated as the conformational determinant for endocytosis (24).

On the other hand, several authors have reported the internalization of the $\alpha_5\beta_1$ receptor. Pioneering studies used fibronectin-coated beads that were endocytosed by cells (31, 32). More recently, direct evidence that the $\alpha_5\beta_1$ fibronectin receptor could be internalized and recycled was provided by electron microscopy and labeling reagent studies (33, 16). This internalization is a constitutive process which occurs even in the absence of ligand similarly to what has been described for the LDL receptor (17). The internalization of the LDL receptor requires a cytoplasmic NPXY sequence (19). This internalization signal adopts a tight turn conformation (21). Similarly, the insulin receptor has two NPXY sequences with a tight turn conformation that are required for internalization (20, 22). In addition the NPXY motif is found in the cytoplasmic domains of at least 10 other cell surface proteins, suggesting that it may play a key role in ligand-independant internalization (19).

Fig.3. Internalization of human β_1 mutants observed by confocal laser scanning microscope after immunofluorescent staining of suspension cells. Harvested cells in suspension were cell surface stained for the human β_1 subunit with the primary K20 mAb anti-human β_1 at 4°C. Then the cells were incubated for 30 min at 37°C to allow internalization, or at 4°C for the controls. The cells were subsequentely fixed, plated onto slides using a cytospin, and permeabilized before incubation with Rhodamine-coupled F(ab)'$_2$ goat anti-mouse IgG. The optical sections into the stained cells (0.6 µm thick) were performed with a Zeiss confocal laser scanning microscope. Bar = 10 µm.

The β_1 subunit contains two NPXY sequences in its cytoplasmic domain, and the NPXY motif around the Tyr $_{783}$ is present in five other integrin β subunits (β_2, β_3, β_5, β_6, and β_7). Therefore it has been proposed that this motif mediates the internalization of integrin receptors. Indeed many integrins, other than the $\alpha_5\beta_1$ receptor, undergo internalization. For instance, the $\alpha_V\beta_5$ integrin regulates receptor-mediated endocytosis of vitronectin (34) whereas endocytosis of fibrinogen into megakaryocytes and platelet α–granules is mediated by the $\alpha_{IIb}\beta_3$ integrin (35). The $\alpha_6\beta_4$ and $\alpha_M\beta_2$ integrins also follow the endocytotic cycle (36).

To address the role of the two NPXY motifs in the internalization of the $\alpha_5\beta_1$ integrin, we have established CHO cell lines expressing NPXY-mutants of the β_1 subunit. In the present study we report that although the $\alpha_5\beta_1$ integrin contains two highly conserved NPXY sequences in the β_1 cytoplasmic domain, these motifs are not required for internalization. Therefore the hypothesis of an internalization of the integrin via clathrin coated pits implies that the internalization signal required for the interaction of $\alpha_5\beta_1$ with adaptor proteins differs from the well-identified signals of the constitutively recycled receptors (24). This new signal would be localized either within the 28 amino acids of the cytosolic tail of the α_5 subunit, or within the membrane proximal 26 amino acid residues of the β_1 cytoplasmic domain, since the variant β_{1B} can be internalized as well. However, no consensus internalization motifs are found in these short domains and the endocytosis mediated by a new type of internalization motif seems unlikely when two well-defined NPXY internalization signals are already present. Therefore, it seems more likely that the internalization of the $\alpha_5\beta_1$ receptor might be due to a continuous non-specific uptake and recycling of the plasma membrane, that would require neither coated pits nor internalization signals recognized by adaptor proteins. This view is substantiated by the very slow rate of the endocytosis of $\alpha_5\beta_1$ (16). Indeed, this rate of internalization is not consistent with the rate of coated pits formation and their turn over, unless it is assumed that the integrins are not concentrated into the coated pits. Furthermore, clathrin and $\alpha_5\beta_1$ receptors do not colocalize *in vivo* (37, 38).

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