

Intracellular Trafficking of CD23: Differential Regulation in Humans and Mice by Both Extracellular and Intracellular Exons¹

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In mouse models of food allergy, we recently characterized a new CD23b-derived splice form lacking extracellular exon 5, $b\Delta 5$, which undergoes constitutive internalization and mediates the transepithelial transport of free IgE, whereas classical CD23b is more efficient in transporting IgE/allergen complexes. These data suggested that regulation of endocytosis plays a central role in CD23 functions and drove us to systematically compare the intracellular trafficking properties of human and murine CD23 splice forms. We found that CD23 species show similar endocytic behaviors in both species; CD23a undergoes constitutive clathrin-dependent internalization, whereas CD23b is stable at the plasma membrane. However, the mechanisms controlling these similar behaviors appeared to be different. In mice, a positive internalization signal was localized in the cytoplasmic region shared by all CD23 splice forms. This positive signal was negatively regulated by the intracellular CD23b-specific exon. In addition, the fact that alternative splice forms lacking exons of the extracellular region (5, 6, 7, and/or 8) were all constitutively internalized suggested that endocytosis of murine CD23 is regulated by a process similar to the outside-in signaling of integrins. In humans, the internalization signal was mapped in the CD23a-specific intracellular exon. Interestingly, this signal also behaved as a basolateral targeting signal in polarized Madin-Darby canine kidney cells. The latter result and the fact that human intestinal cell lines were found to coexpress both CD23a and CD23b provide a molecular explanation for the initial observations that CD23 was found at the basolateral membrane of intestinal epithelial cells from allergic patients. *The Journal of Immunology*, 2005, 174: 5562–5572.

The low affinity receptor for IgE (CD23) represents a unique case in the large family of Ig receptors, because it is formed by a single protein that does not belong to the Ig superfamily (1, 2). It is also quite different from the high affinity IgE receptor, because it is not specifically expressed in cells directly involved in the allergic reaction, but has been found to be expressed on a growing number of cell types, including epithelial cells (3–5). The exact function of CD23 in the allergic process remained poorly understood; the CD23 knockout mice showed only an Ag-specific IgE-mediated response defect, and transgenic mice exhibited reduced serum IgE levels (6, 7). However, recent data provided direct evidence that CD23 indeed plays a central role in the allergic reaction that is responsible for the rapid transepithelial transport of IgE/allergen complexes and subsequent delivery of intact Ags to mast cells in the subepithelial compartment (5, 8, 9).

The CD23 molecule is a type II transmembrane glycoprotein composed of a N-terminal short cytoplasmic tail, a single trans-

membrane domain, a coil-coiled domain (also called stalk region) responsible for high affinity IgE binding, and the C-terminal lectin-like IgE binding domain (1, 2, 10, 11). The two classical CD23 splice forms, *a* and *b*, are generated by the use of alternative transcription initiation sites and have been described in both mice and humans (3, 12, 13). These two classical splice forms differ only by the first N-terminal amino acids of the intracytoplasmic region, seven for *a* and six for *b*, and show differential expression pattern and functions.

In humans, the expression of CD23a has been suggested to be restricted to B lymphocytes, whereas the expression of CD23b can be induced in various cell types by treatment with IL-4 and/or LPS (3, 14). The two CD23 species mainly differ by their intracellular trafficking properties; although CD23a undergoes efficient endocytosis, CD23b is not efficiently internalized, but is able to mediate phagocytosis of IgE-opsonized particles when expressed in macrophages (15).

In mice, CD23a shows a similar expression pattern as its human counterpart (13). The real expression of CD23b remained controversial until recent studies showing that it is effectively expressed in intestinal epithelial cells from sensitized mice *in vivo* or is induced by IL-4 *in vitro* (8, 9). The functional differences between CD23a and CD23b in mice were therefore even less clear than in the human model. However, our most recent studies established that CD23b is expressed at the apical membrane of intestinal cells from where it is involved in the apical to basolateral transport of IgE/allergen complexes (8, 9). In addition, we characterized a new splice form derived from CD23b that is induced in intestinal cells by IL-4 *in vitro* and sensitization *in vivo* (8, 16). This new CD23b-derived splice form lacking exon 5 ($b\Delta 5$) of the extracellular stalk region is constitutively internalized, in contrast to classical CD23b (8), and mediates the apical to basolateral transport of free IgE

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(16). The latter results suggested that in contrast to what was suggested by the human model, murine CD23b-derived splice forms can be efficiently internalized and therefore involved in IgE transport events. They also suggest that endocytosis of CD23b in mice is tightly regulated by the extracellular domain *in vivo*.

The CD23 splice forms appear, then, to be involved in various endocytic pathways, including clathrin-mediated endocytosis (human CD23a and murine *bΔ5*), phagocytosis of IgE-opsonized particles (human CD23b), and transcytosis of IgE and IgE/allergen complexes (murine CD23b and *bΔ5*) (8, 15, 16). However, the mechanisms involved in the differential endocytosis of CD23 splice forms remain poorly characterized, with only one study in the human model (15). In this study the molecular mechanisms controlling endocytosis of murine and human CD23 species were investigated using various tools that we have recently developed to characterize endocytosis of the murine *bΔ5* splice form (8, 16). The results obtained indicate that murine and human CD23a and CD23b splice forms show the same overall endocytic properties. However, identification of the determinants required for endocytosis reveals that the mechanisms controlling the trafficking of CD23 are completely different in the two species. The differences between the two species were further exemplified by analysis of the expression pattern of CD23 splice forms in human intestinal cell lines. We found that in humans, in contrast to what we previously found in mice, intestinal cells coexpress CD23a and CD23b, and the two splice forms show different localizations in polarized cells.

Materials and Methods

Cell culture and transfection

The HeLa human epithelial (American Type Culture Collection), Madin-Darby canine kidney (MDCK)³ strain II canine renal epithelial (gift from A. Zahraoui, Curie Institute, Paris, France), and HT29 human intestinal epithelial (gift from Dr. M. Heyman, Necker Hospital, Paris, France) cell lines were grown in DMEM supplemented with 10% FBS, 20 mM L-glutamine, and 5 mg/ml streptomycin sulfate (Invitrogen Life Technologies). The human T84 intestinal epithelial cell line (gift from Dr. M. Heyman) was grown in mixed medium containing equivalent amount of DMEM and Ham's F-12 (Invitrogen Life Technologies) supplemented with 10% FBS, 200 mM HEPES (Invitrogen Life Technologies), 1% essential amino acids, 20 mM L-glutamine, and 5 mg/ml streptomycin sulfate. MDCK, HT29, or T84 cells monolayers were obtained by seeding 1 million cells on a 12-mm diameter, 0.4-μm pore size Transwell (Costar) for 7 days.

For transient transfections, HeLa cells were grown 1 day before transfection in six-well plates directly on coverslips for fluorescence microscopy studies. Cells were then transfected with a maximum of 5 μg of plasmids/well using the calcium phosphate transfection kit (Invitrogen Life Technologies) and were processed for endocytosis studies the following day. Stable MDCK cell lines were obtained by transient transfection of CD23 encoding plasmids as previously described (16). Selection was started 1 day after transfection by adding 0.6 mg/ml Geneticin (Invitrogen Life Technologies).

For clathrin adaptor protein complex 2 (AP-2) knockdown assays, HeLa cells were transfected with small interfering RNA (siRNA) duplex (Dharmacon) specific for the human μ2 subunit of the clathrin adaptor complex AP-2³ or luciferase as a negative control with Oligofectamine according to the manufacturer's instruction (Invitrogen Life Technologies; μ2 siRNA: 5'-AAG UGG AUG CCU UUC GGG UCA-3'; luciferase siRNA: 5'-CGU ACG CGG AAU ACU UCG ATT-3') (17). Briefly, 200 pmol of siRNA was transfected the first day. On the second day, cells were transfected with CD23 encoding plasmids, incubated for ~6 h, then washed twice in PBS and again transfected with 200 pmol of siRNA. Cells were processed for endocytosis assays on the third day.

RNA extraction and RT-PCR

RT-PCR was performed on total RNA extracted from T84 or HT29 cells using the RNeasy Mini kit (Qiagen). Purified RNA was analyzed by electrophoresis to check its integrity and quantified by measuring absorbance at 260 nm. Total RNA (1 μg) was subjected to RT-PCR using the One Step RT-PCR kit from Qiagen and following the manufacturer's instructions. Briefly, 0.5 μg of RNA was added to a final 50 μl of mix containing dNTP (0.4 mM each), 10 μl of Q solution, 2 μl of enzymes mix, 1× buffer, and 0.3 mM of each primer (CD23a-specific upper: hCDa, 5'-CAC AAT GGA GGA AGG TCA ATA TTC AG-3'; CD23b-specific upper: hCD23b, 5'-ATT TAG CAC AAT GAA TCC TCC AAG CCA GG-3'; hCD23a and hCD23b common lower, 5'-TTG AGA GAC GTT CCG GGC AGC CTT CTC TTC CAG CTG TT-3'; primers specific for coiled-coil: upper hCC, 5'-GGC ACT GGG ACA CCA CAG ACA GTC TAA AAC A-3'; lower hCC, 5'-AAA TCT GAA GCT TCG TTC CTC TCG TTC AAT T-3'; ribosomal RNA 18S: upper, 5'-CGG CTA CCA CAT CCA AGG AA-3'; and lower, 5'-GCT GGA ATT ACC GCG GCT-3'). This mix was then subjected to RT-PCR by programming a DNA thermal cycler (GeneAmp, PCR system 2700; Applied Biosystems) to perform a protocol as follows: 54°C for 30 min for one cycle; 95°C for 15 min for one cycle; 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s for 40 cycles; and 72°C for 7 min for final extension. For each primer pair, control amplifications were performed in which cDNA was omitted from the RT-PCR. RT-PCR products were analyzed by electrophoresis (1.5% agarose) using the 1-kb plus DNA ladder m.w. marker from Invitrogen Life Technologies.

DNA constructs

The murine CD23 splice forms *a*, *b*, *bΔ5*, and *bΔ6* subcloned in the pCR3.1 vector (Invitrogen Life Technologies) were described previously (8, 16). The *bΔ5,6* and *bΔ5,6,7* splice forms subcloned in the pCR3.1 vector were obtained using the same method as that described for *bΔ5* and *bΔ6* (8). The GFP-tagged 2xYFVE endosomal marker (18) and the dynamin wild-type and dominant negative mutant constructs (19) were gifts from H. Stenmark (Norwegian Radium Hospital, Oslo, Norway) and S. L. Schmid (Scripps Research Institute, La Jolla, CA), respectively. The GFP-tagged Eps15 constructs were described previously (20).

MCY and MTM CD23 mutants were obtained by PCR using primers designed to shorten the N-terminal intracytoplasmic region. The mutations were introduced by PCR using MCY (5'-C AGA ATG GGA TAC TGG GAA CCT CCT AGA-3') or MTM (5'-C AGA ATG AGA CGT GGG ACA CAG CTC-3') upper primers together with the common downstream primer Oligo-E (5'-GGA GCC CTT GCC AAA ATA GTA GCA C-3'). The generated PCR fragments were then cloned into pCR3.1 plasmid using the TA cloning kit (Invitrogen Life Technologies), and full-length MCY and MTM constructs were obtained by replacing the corresponding 5' region of wild-type CD23b using appropriated restriction sites.

Chimeric human/mouse CD23 constructs aLH and bLH were obtained by PCR using a three-step protocol.

Step 1. The 5' region of human CD23a and CD23b cDNAs were obtained by RT-PCR performed on RNA from a human B cell line (Alf; gift from F. Le Deist, Hopital Necker, Paris, France) using upper primers specific for human CD23a or CD23b (hCD23a specific upper, 5'-C ACA ATG GAG GAA GGT CAA TAT TCA G-3'; hCD23b specific upper, 5'-A TTT AGC ACA ATG AAT CCT CCA AGC CAG G-3') with LOWLH (5'-GCA GTT CCG CTG GAC ACC TGC AA-3') as a human/mouse chimeric lower primer. Mouse IgE binding domain encoding sequence was amplified by PCR using UPLH (5'-TGT CCA GCG GAA CTG CAT GCA AC-3') as a human/mouse chimeric upper primer that partially match with the LOWLH primer and with LOWMOUSE (5'-TCA GGG TTC ACT TTT TGG GGT GGG-3') as a lower primer.

Step 2. The two PCR products obtained at step 1 were then annealed and used as their own template to synthesize complete chimeric cDNA by PCR (without primers) by programming the thermocycler as followed: 94°C for 5 min for one cycle, 94°C for 30 s, 50°C for 30 s, and 68°C for 45 s for five cycles, and a final extension step at 68°C for 7 min.

Step 3. A third PCR was then performed to amplify chimeric constructs on 1 μl of product from step 2 with upper primers specific for human CD23a or CD23b together with the lower primer LOWMOUSE. Those two constructs were then cloned in pCR3.1 plasmid as previously described. MCYLH and MTMLH chimeric CD23 mutants were obtained by PCR using primers designed to shorten the human N-terminal intracytoplasmic region. The mutations were introduced by amplifying the 5' region of human CD23 PCR using MCYLH (5'-C ACA ATG GAG ATC GAG GAG CTT CCC-3') or MTMLH (5'-C ACA ATG AGG CGT GGG ACT CAG ATC-3') upper primers together with the common downstream primer LOWLH. The generated PCR fragments were then cloned into

³ Abbreviations used in this paper: MDCK, Madin-Darby canine kidney; AP-2, clathrin adaptor protein complex 2; siRNA, small interfering RNA; CCP, clathrin-coated pit.

pCR3.1 plasmid, and full-length MCYLH and MTMLH constructs were obtained by replacing the corresponding 5' region of wild-type CD23b using appropriated restriction sites. The sequence of all CD23 constructs generated by PCR was confirmed by nucleotide sequencing (sequencing facility, Cochin Institute).

Immunofluorescence

The internalization of CD23 was characterized by fluorescence microscopy, after the intracellular accumulation of plasma-membrane bound anti-CD23 Ab (B3B4, rat monoclonal IgG2a) (21) or anti-DNP mouse monoclonal IgE (Clone Spe-7; Sigma-Aldrich). The endosomal localization of the internalized Abs was followed using transferrin as a marker of early endosomes as previously described (8, 16). Briefly, HeLa cells transfected with CD23 encoding plasmids were first incubated for 20 min at 37°C in DMEM to eliminate receptor-bound endogenous transferrin, washed in cold PBS, and then incubated for 1 h at 4°C in the presence of B3B4 (50 µg/ml) in PBS supplemented with BSA (Sigma-Aldrich) at 1 mg/ml (PBS-BSA) or of IgE (10 µg/ml) in PBS-BSA supplemented 1 mM CaCl₂ and 1 mM MgCl₂ (IgE binding buffer). Cells were then washed twice in DMEM-BSA (1 mg/ml) and incubated for 30 min at 37°C in DMEM-BSA with or without 6 µg/ml Alexa 594-conjugated transferrin (Molecular Probes). The cells were rapidly cooled to 4°C using cold DMEM-BSA, washed twice in cold PBS, fixed with 4% paraformaldehyde and 0.03 M sucrose at 4°C for 30 min, and quenched with 50 mM NH₄Cl in PBS at room temperature for 10 min. To reveal internalized anti-CD23 Abs, cells were incubated for 30 min at room temperature in the presence of an Alexa 488-conjugated goat anti-rat IgG secondary Ab (Molecular Probes) in a permeabilizing buffer (PBS-BSA and 0.1% Triton). To reveal internalized IgE, cells were permeabilized and incubated for 30 min in the presence of a rat anti-mouse IgE Ab (1/50; Southern Biotechnology Associates) and then with an Alexa 488-conjugated goat anti-rat IgG secondary Ab (Molecular Probes). Cells were then washed twice in PBS and mounted on microscope slides in PBS/glycerol (50/50). The percentage of endocytosis was calculated as the number of CD23-expressing cells showing an intracellular B3B4 staining colocalizing with internalized transferrin for 100 counted CD23-expressing cells.

To characterize the endosomal localization of CD23 at steady state, HeLa cells were transiently transfected with both CD23 and GFP-2xYFVE encoding plasmids. Cells were fixed as described above and incubated for 30 min at room temperature in the presence of the anti-CD23 mAb in permeabilization buffer. Cells were then washed twice and incubated in PBS-BSA with an Alexa 594-conjugated goat anti-rat IgG secondary Ab (Molecular Probes).

To test the ability of chimeric CD23 constructs to bind murine IgE, HeLa cells transfected with aLH- or bLH-encoding plasmids were washed once in PBS and incubated with the murine anti-DNP monoclonal IgE (1/100) for 1 h at 4°C in IgE binding medium. Cells were washed, fixed, and stained with a rat anti-mouse IgE Ab (1/50; Southern Biotechnology Associates) in PBS-BSA revealed using an Alexa 488-conjugated goat anti-rat IgG secondary Ab. Cells were finally washed twice in PBS and mounted on microscope slides in PBS/glycerol (50/50).

Samples were examined under an epifluorescence microscope (Leica; Wetzlar) attached to a cooled CCD camera (Micromax; Princeton Instruments). The pictures were taken using MetaMorph (Universal Imaging), and the final images were obtained using National Institutes of Health Image (<http://rsb.info.nih.gov/nih-image/>) and Photoshop (Adobe Systems).

Expression and distribution of CD23 chimeras in polarized MDCK cells

To characterize the steady-state distribution of CD23 chimeras in polarized cells, MDCK cell lines stably expressing aLH, bLH, or MCYLH were grown on Transwell filters for 6 days. The monolayers were washed, fixed, and stained for 1 h at room temperature with the B3B4 anti-mouse CD23 Ab and a rabbit anti-ZO-1 polyclonal Ab (Zymed Laboratories) in permeabilization buffer. Cells were washed twice, and primary Abs were revealed using an Alexa 488-conjugated goat anti-rat IgG (Molecular Probes) and a Cy3-conjugated donkey anti-rabbit IgG Ab (Jackson ImmunoResearch Laboratories) as described above. The MDCK monolayers were finally washed twice in PBS, and filters were mounted between microscope slides and coverslips in PBS/glycerol (50/50). All samples were analyzed by confocal microscopy (TCS SP2 AOBIS; Leica), and the final images were obtained using NIH Image and Adobe Photoshop.

CD23 internalization indicated by flow cytometry

The intracellular accumulation of membrane-bound PE-conjugated B3B4 (BD Biosciences) was quantified by flow cytometry as described in our recent study (16). Cells were maintained adherent during the assay; incubations and washes were conducted in plates. HeLa cells treated, or not, with siRNA and transfected with CD23-encoding plasmids were washed once in PBS and incubated with B3B4-PE (1/100) for 1 h at 4°C in PBS-BSA (10 mg/ml). After two washes in cold PBS-BSA, the cells were allowed to internalize by incubation at 37°C for 0, 30, or 60 min in DMEM-BSA (10 mg/ml), then rapidly cooled on ice. After two washes in cold PBS, the remaining plasma membrane-associated B3B4-PE was cleaved by a 4-min incubation in the presence of trypsin (0.05% in PBS) at 37°C. Plates were then rapidly cooled on ice, cold PBS-BSA was added to each well, and cells were resuspended by pipetting up and down. The cells were finally washed by harvesting at 2500 rpm for 5 min at 4°C, resuspended in cold PBS, and analyzed by flow cytometry (flow cytometry facility, Cochin Institute). Endocytosis levels after 30- and 60-min internalization were calculated as the percentage of initial B3B4 binding (no internalization, no trypsin) after removing the mean PE fluorescence at time zero (no internalization, trypsin). The data (mean ± SE) presented are the values obtained from at least three independent experiments.

Results

Endocytosis of murine CD23b is controlled by the extracellular stalk region

Our previous results established that murine intestinal cells express CD23b (8, 9, 16). Moreover, by systematic sequencing of RT-PCR products, we also identified two new CD23b-derived splice forms lacking exons 5 (*bΔ5*) or 6 (*bΔ6*) of the extracellular stalk domain (8) (Fig. 1). Interestingly, those splice forms showed efficient internalization in contrast to classical CD23b (8, 16) (Fig. 2E). Thus, although CD23b and new splice forms share the same cytoplasmic tail (Fig. 1), the extracellular stalk domain appeared to regulate the internalization of CD23b. In this study we report the identification

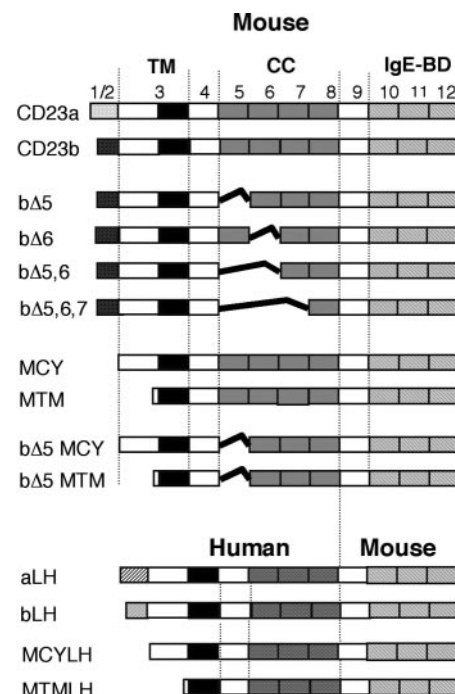


FIGURE 1. Schematic representations of CD23 splice forms and mutants. The functional exonic organization of classical murine *a* and *b* CD23 splice forms as well as that of all the new murine CD23b-derived splice forms, *bΔ5*, *bΔ6*, *bΔ5,6*, and *bΔ5,6,7*, are indicated. The organization of the mutant forms of murine CD23 and that of the chimeric human/mouse CD23 constructs are also shown. TM, transmembrane; CC, coiled-coil domain; IgE-BD, lectin-like IgE binding domain.

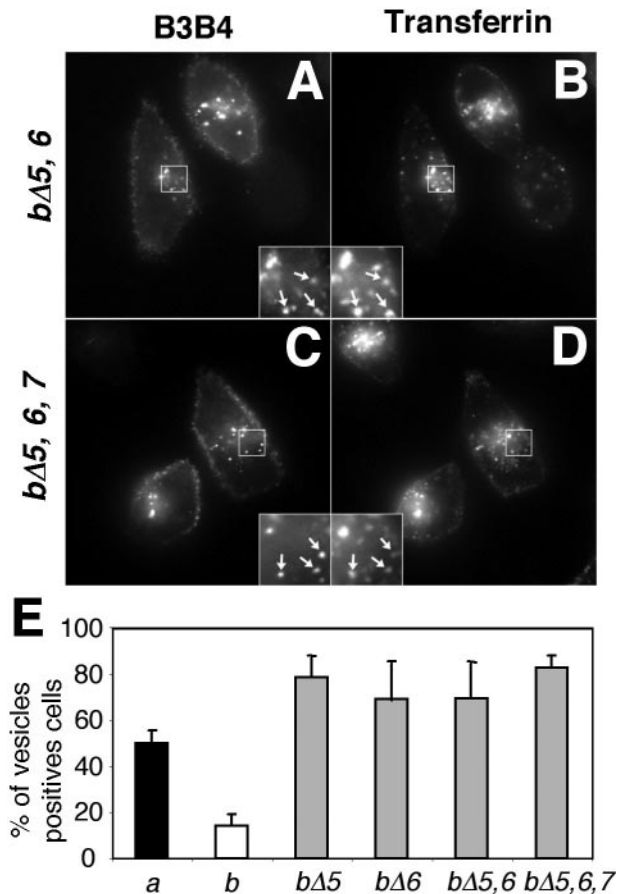


FIGURE 2. The *bΔ5,6* and *bΔ5,6,7* splice forms are efficiently internalized. *A–D*, HeLa cells transiently transfected with plasmids encoding *bΔ5,6* (*A* and *B*) or *bΔ5,6,7* (*C* and *D*) splice forms were incubated with the B3B4 anti-CD23 mAb for 1 h at 4°C, washed in cold PBS, and incubated at 37°C for 30 min to allow internalization of the membrane-bound Abs in the presence of Alexa 594-labeled transferrin to stain early endosomes. Cells were then washed, fixed, and permeabilized, and intracellular B3B4 Ab was revealed using an anti-rat Alexa 488-labeled secondary Ab. *A* and *C*, Green fluorescence emitted by Alexa 488 (CD23). *B* and *D*, Red fluorescence emitted by Alexa 594 (transferrin). *Insets* show higher magnifications of representative areas. Arrows stress colocalizing CD23 and transferrin dots. *E*, HeLa cells transfected with plasmids encoding CD23 *a*, *b*, *bΔ5*, *bΔ6*, *bΔ5,6*, and *bΔ5,6,7* splice forms were examined for B3B4 uptake as indicated above. Internalization was calculated as the percentage of cells showing CD23 staining colocalizing with transferrin in 100 CD23-expressing cells. The data (mean ± SE) presented in this figure are the means of at least three independent experiments.

of two additional new CD23b-derived splice forms lacking exons 5 and 6 (*bΔ5,6*) or exons 5, 6, and 7 (*bΔ5,6,7*) of the stalk domain (Fig. 1). These splice events were identified by systematic sequencing of RT-PCR products designed to amplify full-length CD23b from the murine intestinal cell line IEC-4. Clones encoding *bΔ5,6* and *bΔ5,6,7* were found only once among the ~100 individual clones sequenced, suggesting that they are unlikely to represent relevant functional splice forms in vivo.

We decided to investigate their endocytic properties to complete our analysis of the role of the stalk domain in this process. Upon transient expression in HeLa cells, both *bΔ5,6* and *bΔ5,6,7* were correctly addressed at the plasma membrane and seemed to be correctly folded, as indicated by the specific staining observed using the anti-CD23 Ab B3B4 (data not shown). In addition, as shown in Fig. 2, HeLa cells expressing either *bΔ5,6* (*A* and *B*) or

bΔ5,6,7 (*C* and *D*) efficiently internalized membrane-bound anti-CD23 Abs, as shown by the extensive colocalization of the CD23 staining with internalized transferrin after 30-min incubation at 37°C (*A–D*, *insets*). To better compare endocytic abilities of CD23 splice forms including *a*, *b*, *bΔ5*, *bΔ6*, *bΔ5,6*, and *bΔ5,6,7*, we calculated the percentage of CD23-expressing cells showing colocalization of anti-CD23 staining with internalized transferrin (Fig. 2*E*). Using this semiquantitative technique, CD23a showed efficient internalization compared with CD23b, which showed background internalization levels (Fig. 2*E*). In contrast, all the new CD23b splice forms presenting deletions of exons of the stalk region were internalized even more efficiently than CD23a. According to structural differences between classical CD23b and new splice forms, these results suggest that the extracellular stalk region negatively regulates endocytosis of murine CD23b.

Endocytosis of murine CD23 is driven by determinants present in the cytoplasmic region shared by all CD23 splice forms

The results presented above showing efficient internalization of the new CD23b splice forms suggested that the cytoplasmic domain of classical CD23b shared by all these splice forms might contain a motif(s) for endocytosis. They also suggested that this putative motif(s) were under the control of the extracellular stalk region. To characterize this putative internalization motif, mutants of the cytoplasmic region were generated (Fig. 1) in which N-terminal exon specific for CD23a or CD23b (MCY) or almost all the cytosolic tail (MTM) were deleted.

The endocytic behavior of the CD23 mutants was then analyzed using the semiquantitative internalization assay described above. As shown in Fig. 3, the MCY mutant was highly efficiently internalized compared with CD23b (*A*, *B*, and *E*). This unexpected result suggested that CD23b-specific exon negatively regulates endocytosis of CD23b (see *Discussion* for details). It also suggested that endocytic determinants were likely to reside in the cytoplasmic domain shared by all CD23 splice forms. This latter hypothesis was confirmed by the fact that the MTM mutant was very poorly internalized, as evidenced by its diffuse staining at the plasma membrane after 30 min at 37°C (Fig. 3, *C–E*). Identical results were obtained when murine IgE were used as a ligand to follow endocytosis of CD23 splice forms and mutants. Indeed, as shown in Fig. 4, IgE were efficiently internalized in cells expressing the MCY mutant (*A* and *B*), but remained on the plasma membrane in cells expressing the MTM mutant (*C* and *D*) or wild-type CD23b (not shown). A quantitative analysis of the results indicates that results obtained with IgE are very similar to those obtained using the anti-CD23 Ab (compare Figs. 4*E* and 3*E*). These results are in agreement with our previous findings showing a strong correlation between results obtained with anti-CD23 Abs and IgE (8, 16) and also show that the anti-CD23 mAb B3B4 could be used as a ligand to characterize endocytosis of CD23.

The semiquantitative results obtained in Fig. 3 were confirmed using a flow cytometry-based assay designed to directly quantify intracellular accumulation of membrane bound anti-CD23 Ab (see *Materials and Methods* for details) (16). Results obtained with this assay (Fig. 5) clearly confirmed those obtained with the semiquantitative assay and showed that endocytic rates of the MCY mutant and CD23a were very similar and approximately three times higher than those of the MTM and CD23b. Similar results were obtained for mutants of *bΔ5* presenting the same deletions within the cytoplasmic tail (*bΔ5MCY* and *bΔ5MTM*; Fig. 1), showing that internalization of *bΔ5* is also dependent of the same cytoplasmic region (data not shown).

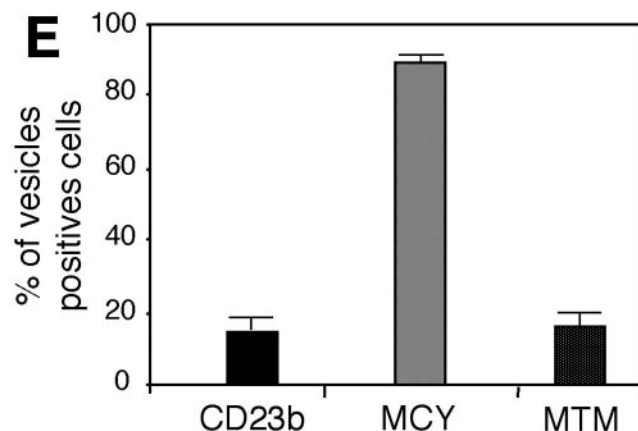
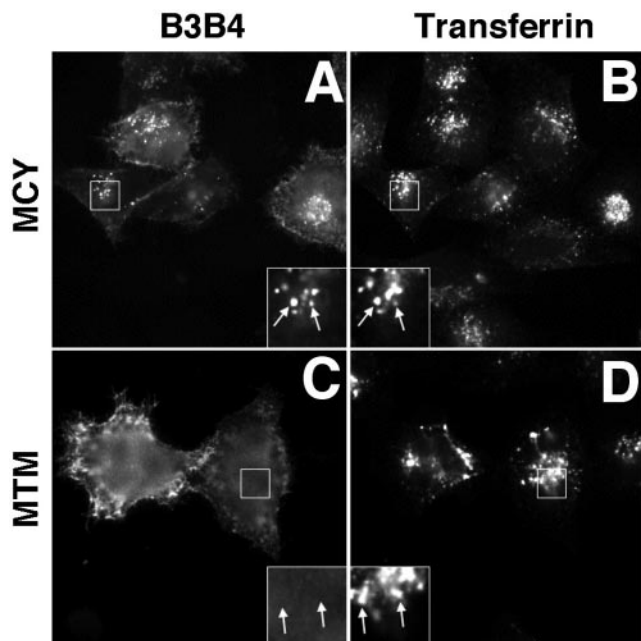


FIGURE 3. Endocytic signal is present in the cytoplasmic region shared by all murine CD23 splice forms. *A–D*, HeLa cells transiently transfected with plasmids encoding MCY (*A* and *B*) or MTM (*C* and *D*) mutants were examined for B3B4 uptake as indicated in Fig. 2. *A* and *C*, Green fluorescence emitted by Alexa 488 (CD23). *B* and *D*, Red fluorescence emitted by Alexa 594 (transferrin). *Insets* show higher magnifications of representative areas. Arrows stress colocalizing CD23 and transferrin dots. *E*, Internalization efficiency of the MCY and MTM mutants was calculated as described in Fig. 2*E*. The data (mean $\pm$ SE) presented in this figure are the means of at least three independent experiments.

Murine CD23 is constitutively internalized through clathrin-coated pits (CCPs)

The two assays used to follow endocytosis of CD23 are based on the use of prebound Abs. Therefore, it was not possible to formally exclude that the binding of anti-CD23 Abs or IgE induced the observed internalization of CD23 by conformational changes and/or aggregation. To eliminate such a possibility, the steady-state intracellular localization of the MCY mutant was compared with that of markers of endosomes. Indeed, if internalization of MCY were a constitutive process, it was expected to localize in endosomes in the absence of any extracellular ligand. As expected, the MCY mutant was found to colocalize with endosomal markers at steady state, indicating that endocytosis of CD23 is constitutive (data not shown; see Fig. 9 for human CD23).

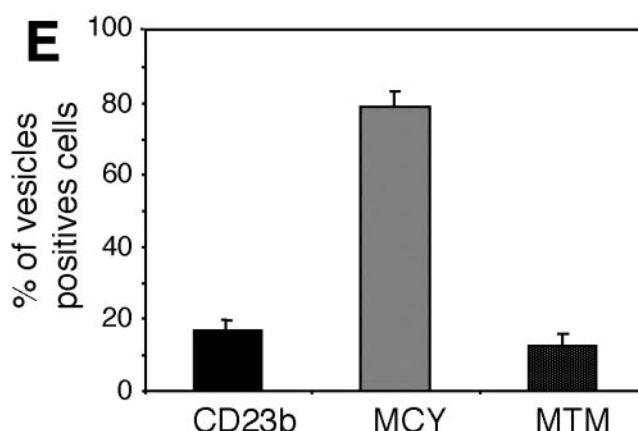
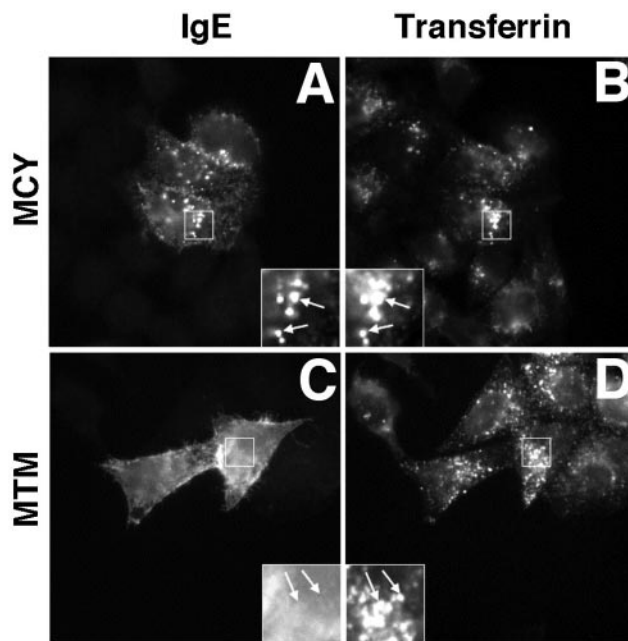


FIGURE 4. Endocytosis of IgE by CD23 splice forms and mutants. *A–D*, HeLa cells transiently transfected with plasmids encoding MCY (*A* and *B*) or MTM (*C* and *D*) mutants were examined for IgE uptake as indicated in *Materials and Methods* and under the same conditions as described in Fig. 3. *A* and *C*, Green fluorescence emitted by Alexa 488 (IgE). *B* and *D*, Red fluorescence emitted by Alexa 594 (transferrin). *Insets* show higher magnifications of representative areas. Arrows stress colocalizing IgE and transferrin dots. *E*, Internalization efficiency of CD23b and of the MCY and MTM mutants was calculated as described in Fig. 3*E*. The data (mean $\pm$ SE) presented in this figure are the means of at least three independent experiments.

The flow cytometry-based assay was then used to investigate the endocytic pathway, followed by murine CD23. We first tested inhibitors of the clathrin-dependent pathway, which is the major pathway for constitutive internalization of plasma membrane transmembrane proteins. Expression of Eps15 and dynamin dominant negative mutants, two well-characterized inhibitors of clathrin-dependent endocytosis (22), was found to inhibit endocytosis of the MCY mutant by $\sim 40\%$ (data not shown). The involvement of the clathrin-coated vesicle formation in the endocytosis of CD23 was also tested using siRNA against the $\mu 2$ subunit of the AP-2 complex, which plays a central role in the formation of CCPs and, therefore, in clathrin-dependent endocytosis (23). The expression of the AP-2 complex is drastically reduced in $\mu 2$ siRNA-treated cells in which the formation of CCPs and vesicles is

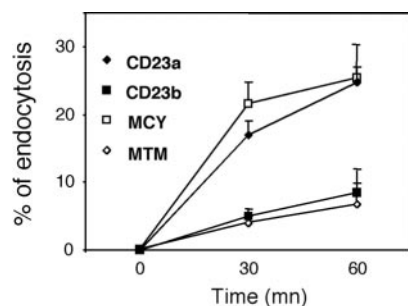


FIGURE 5. Endocytosis of murine CD23 quantified by flow cytometry. HeLa cells transfected with CD23a-, CD23b-, MCY-, or MTM-encoding plasmids were tested for PE-conjugated B3B4 uptake by flow cytometry as described in *Materials and Methods*. Results are expressed as the mean percentage of internal PE fluorescence after trypsin treatment and removal of the value of residual cell surface staining at time zero. The data (mean $\pm$ SE) presented in this figure are the mean values obtained from at least three independent experiments.

strongly inhibited (17). As shown in Fig. 6, endocytosis of MCY mutant as well as murine CD23a, used as a positive control, was inhibited by $\sim$ 50% in AP-2 knocked-down cells compared with control luciferase siRNA-treated cells.

Together these results show that the intracytoplasmic region shared by all murine CD23 splice forms contains determinants for constitutive internalization through CCPs and vesicles.

Human intestinal epithelial cells express both CD23b and CD23a upon polarization

As indicated above and in our previous studies, murine intestinal cells coexpress two different CD23 splice forms, CD23b constitutively and both CD23b and b Δ 5 upon sensitization in vivo and IL-4 in vitro (8, 9, 16). In humans, immunocytochemistry studies indicated that intestinal epithelial cells do express CD23, but the molecular species expressed in these cells were not further characterized (24). With this goal in mind, we designed two sets of primers to amplify the 5' region of human CD23a or CD23b corresponding to exons 1–4 (Fig. 7). In addition, another primer set was designed to amplify the region corresponding to the extracellular stalk domain of human CD23 (exons 4–8) to enable the identification of putative splice events within this domain (exons 5–7) similar to those we found in mouse intestinal cells. The efficiency of all the primer pairs was first tested by amplifying CD23 from human B lymphocyte cell lines (data not shown), and they were

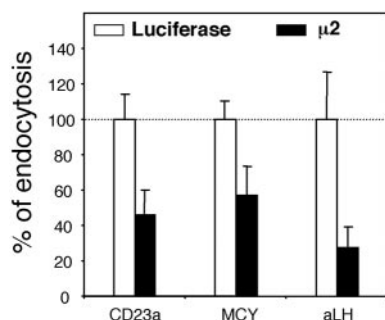


FIGURE 6. Mouse and human CD23 are internalized through clathrin-coated pits. HeLa cells cotransfected with either μ 2- or luciferase-specific siRNA together with CD23a-, MCY-, or aLH-encoding plasmids were tested for B3B4 uptake by flow cytometry as described in Fig. 4. Results are expressed as the mean percentage of endocytosis compared with the control (luciferase, 100%). The data (mean $\pm$ SE) presented in this figure are the values obtained from at least three independent experiments.

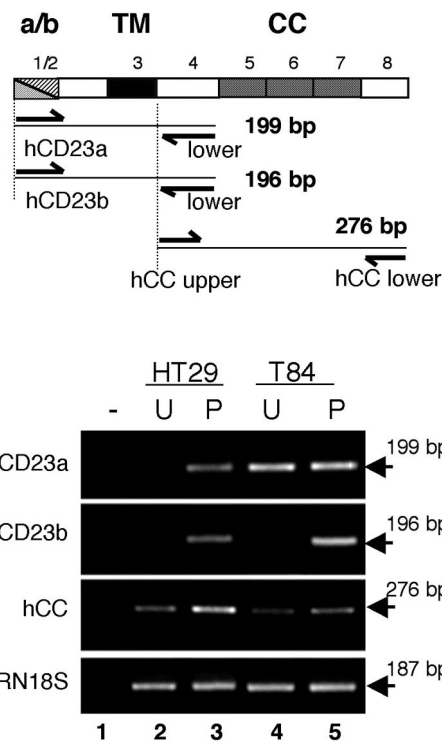


FIGURE 7. Expression of CD23 splice forms by human intestinal cells. The primer couples used to amplify human CD23a, CD23b, and the region corresponding to the coiled-coil or stalk domain are shown. a/b, a- or b-specific exon; TM, transmembrane; CC, coiled-coil or stalk domain. Total RNA were isolated from HT29 (lanes 2 and 3) or T84 (lanes 4 and 5) cells grown on tissue culture plates (U, unpolarized; lanes 2 and 4) or on Transwell filters (P, polarized; lanes 3 and 5). RT-PCR was performed using the primer couples designed to specifically amplify human CD23a or CD23b or to amplify the coiled-coil domain shared by all CD23 splice forms. Control RT-PCR was performed in which cDNA was omitted for the amplification reactions (–; lane 1). The homogeneity of the samples was checked by amplifying the ubiquitous ribosomal 18S RNA. All samples were run on a 1.5% agarose gel together with a m.w. marker. One representative amplification reaction is shown of at least three independent ones.

then used to amplify CD23 species expressed by the human intestinal epithelial cell lines HT29 and T84 by RT-PCR.

The primer set designed to amplify the stalk domain efficiently amplified fragments of the expected size from both HT29 and T84 cells grown in semiconfluent conditions showing that both cell lines expressed CD23 (Fig. 7, lanes 2 and 4, respectively). Similar results were obtained using primer sets designed to amplify the 3' region of human CD23 corresponding to the IgE binding domain (data not shown). However, amplification of the 5' region appeared to be less efficient because, despite detectable expression of CD23, no amplification products could be observed with CD23a- or CD23b-specific primer sets, except in T84 cells, which seemed to express only CD23a at steady state (Fig. 7, lanes 2 and 4). We then examined whether the expression of CD23 could be affected by cellular polarization, as reported for other immune receptors (25). T84 and HT29 cells were grown on Transwell filters for 1 wk, and effective polarization was checked by confocal microscopy after ZO-1 staining (data not shown). Total RNA was extracted, and RT-PCR was performed. In these conditions, the expressions of both CD23a and CD23b were clearly detected in both cell lines (Fig. 7, lanes 3 and 5). This difference was due to higher amounts

of total RNA in the samples corresponding to polarized cells because bands of similar intensities were obtained when the ubiquitous 18S rRNA was amplified under the same conditions.

These results confirmed that human intestinal cells express CD23 and show that they coexpress both CD23a and CD23b. The situation in humans is thus surprisingly different from that in mice, because murine intestinal cells only express CD23b-derived splice forms (8, 16). The expression of *bΔ5*-like splice forms in human intestinal cells was tested by cloning and sequencing the amplification products corresponding to the stalk region (hCC, Fig. 7), similar to what we previously did for murine CD23 (8). We only found clones corresponding to the full-length stalk region, suggesting that in humans the stalk region is not the target of alternative splice events.

Generation and characterization of chimeric human/mouse CD23 constructs

To study endocytosis of human CD23, we choose to generate chimeric molecules in which the IgE binding domain of the human molecule was replaced by the equivalent domain of the murine protein. These chimeric molecules were expected to allow us to use the same endocytosis assays and then to directly compare the results with those obtained for murine CD23. Indeed, the chimeras were expected to be recognized by the anti-mouse CD23 Ab (B3B4) used in all our functional assays, because this Ab is directed against the IgE binding domain of murine CD23. In addition, the use of chimeric molecules and therefore of the tools to detect murine CD23 would avoid any problem linked to the possible expression of endogenous CD23 by the transfected human cell lines.

Chimeric human/mouse CD23 molecules were generated that were composed of an N-terminal part corresponding to the human sequence (exons 1–8) fused to the C terminus of murine CD23 (exons 10–12). In the resulting chimeric molecules, the cytoplasmic, transmembrane, and extracellular stalk domains were of human origin, whereas the IgE binding domain was from mice (Fig. 1). We thus generated chimeric molecules corresponding to human CD23a and CD23b (aLH and bLH, respectively) and mutants of the cytoplasmic tail similar to those generated in the murine system in which CD23a- or CD23b-specific exons (MCYLH) or almost all the tail (MTMLH) were deleted (Fig. 1).

The aLH, bLH, MCYLH, and MTMLH chimeras were then transiently expressed in HeLa cells and tested for cell surface expression as well as for their capacity to bind murine IgE. All constructs were correctly targeted to the plasma membrane, as indicated by the specific cell surface staining observed with the anti-mouse CD23 Ab (data not shown). Similarly, cells transiently transfected with the chimeric CD23 constructs were able to bind murine IgE (Fig. 8, B–D, and data not shown). The cell surface binding of anti-CD23 Abs or murine IgE was specific, because it was not observed for mock-transfected cells (data not shown and Fig. 8A). Together, these data indicate that the chimeric CD23 molecules are correctly addressed to the cell surface, correctly folded, and functional, i.e., able to bind murine IgE.

Human CD23a is constitutively internalized through clathrin-dependent endocytosis

Endocytic properties of human CD23 splice forms were investigated in only one study (15) in which it was suggested that human CD23a is efficiently internalized through CCPs, whereas CD23b mediates phagocytosis of IgE-opsonized particles in macrophages. In the same study, a clathrin-dependent endocytosis signal was suggested to reside in the CD23a-specific exon by the mean of indirect arguments (see *Discussion*). These results were quite dif-

IgE binding

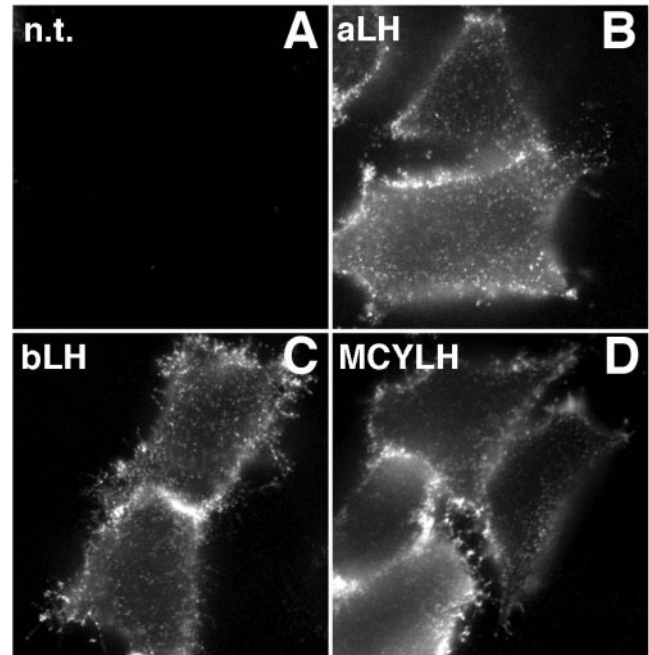


FIGURE 8. Chimeric human/mouse constructs are functional receptors for murine IgE. A–D, HeLa cells transiently transfected with plasmids encoding aLH (B), bLH (C), and MCYLH (D) constructs or mock-transfected (A) were incubated at 4°C for 1 h in the presence of murine monoclonal IgE. Cells were then washed, fixed, permeabilized, and incubated with a secondary rat anti-mouse IgE Ab. Cells were washed again and finally stained with a goat anti rat Alexa 488-conjugated Ab.

ferent from those we found for murine CD23 (see above), and we decided to directly investigate the endocytic properties of human CD23 using the human/mouse chimeras.

Using the immunofluorescence-based assay, we observed that aLH was able to internalize prebound anti-CD23 Abs, as indicated by the colocalization observed between internalized anti-CD23 and transferrin (Fig. 9, A and B, *insets*, arrows). As expected, bLH was not internalized in the same conditions, remaining diffusely distributed on the plasma membrane (Fig. 9, C and D). These observations were confirmed by the quantitative analysis of these experiments stressing that CD23a is 10 times more efficiently internalized than CD23b (Fig. 10A). In addition, we investigated whether endocytosis of CD23a was constitutive or if it could correspond to an induced process due to the binding of anti-CD23 Abs. The intracellular distribution of CD23a at steady state was compared with that of GFP-2xFYVE, a chimeric construct that stains early/sorting endosomes (18). As shown in Fig. 9, the aLH chimera was found in perinuclear vesicular structures at steady state (Fig. 10E), which showed extensive colocalization with the GFP2xFYVE construct (Fig. 10, E and F, *insets*). These results show that the aLH chimera is localized in endosomes at steady state, indicating that CD23a undergoes constitutive internalization.

Based on the results obtained by Yokota et al. (15), the involvement of the clathrin-dependent endocytic pathway in the internalization of aLH was directly tested using siRNA against the AP-2 complex. As expected, internalization of aLH was drastically inhibited in AP-2 knockdown cells (80% inhibition) compared with control luciferase siRNA-treated cells (Fig. 6).

These results show that the chimeric CD23a molecule undergoes efficient and constitutive internalization. They confirm and

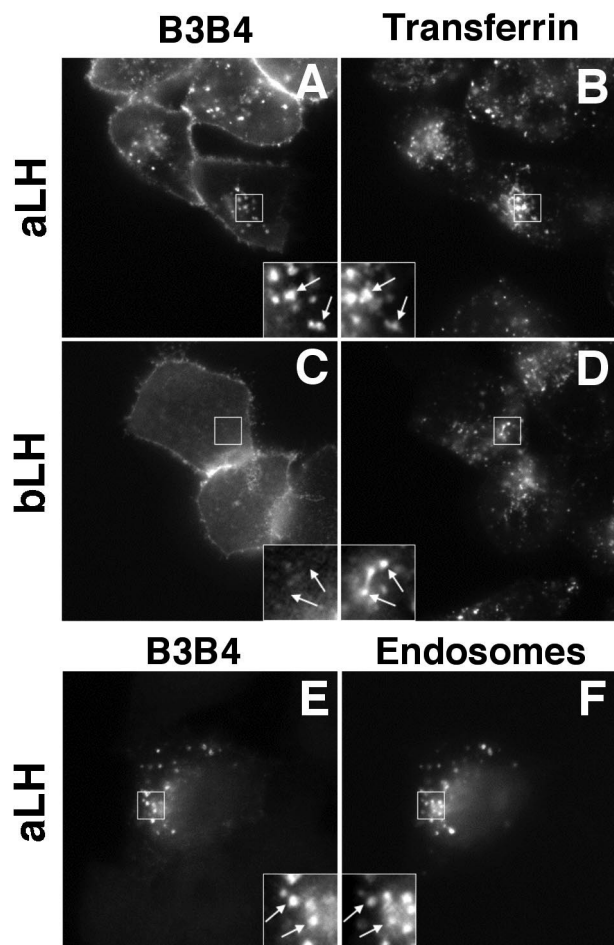


FIGURE 9. aLH, but not bLH, is constitutively internalized. *A–D*, HeLa cells transiently transfected with aLH-encoding (*A*, *B*, *E*, and *F*) or bLH-encoding (*C* and *D*) plasmids were tested for B3B4 uptake as indicated in Fig. 2. *A* and *C*, Green fluorescence emitted by Alexa 488 (CD23). *B* and *D*, Red fluorescence emitted by Alexa 594 (transferrin). *Insets* show higher magnifications of representative areas. Arrows stress colocalizing CD23 and transferrin dots. *E* and *F*, HeLa cells transiently transfected with aLH-encoding plasmid together with GFP-2xFYVE (endosomal marker)-encoding plasmid were washed, fixed, permeabilized, and stained with rat anti-mouse CD23 Ab. Cells were then washed, and CD23 staining was revealed by incubation with a goat anti-rat Alexa 594 secondary Ab. *E*, Red fluorescence emitted by Alexa 594 (CD23). *F*, Green fluorescence emitted by GFP (2xFYVE). *Insets* show higher magnifications of representative areas. Arrows stress colocalizing CD23 and transferrin dots.

extend those obtained by Yokota et al. (15) for human CD23a and indicate that the CD23 chimeras behave in the same way as endogenous proteins and represent useful tools to follow CD23 functions.

Role of *a*- and *b*-specific exons in the regulation of endocytosis of human CD23

The determinants responsible for constitutive clathrin-dependent internalization of human CD23 were investigated using the two mutants of the cytoplasmic region, MCYLH and MTMLH (Fig. 1). The two mutants were transfected in HeLa cells, and their ability to internalize membrane-bound anti-CD23 Abs was analyzed as described in Fig. 9 (not shown) and compared with that of wild-type aLH and bLH chimeras. As indicated by the quantitative analysis of the results (Fig. 10A), the MCYLH mutant was much less efficiently internalized than aLH. This decreased internalization

efficiency was confirmed using the flow cytometry-based assay (Fig. 10B). These results suggested that the *a*-specific exon is required for efficient internalization. In addition, the fact that the MTMLH construct showed the same internalization efficiencies as MCYLH (Fig. 10A and data not shown) indicates that the cytoplasmic region shared by all human CD23 splice forms does not contain additional determinants for endocytosis.

Interestingly, using the two different assays for endocytosis, we repeatedly observed that the bLH chimera was very poorly internalized, if at all (Fig. 10), in agreement with the fact that it remained on the plasma membrane after 30-min incubation at 37°C (Fig. 9C). The internalization levels observed for bLH were strikingly very low and even lower than those for the MCYLH and MTMLH mutants (Fig. 10). The latter observation was surprising, because the level of endocytosis observed for MTMLH was likely to represent background bulk-flow endocytosis due to the lack of any possible intracytoplasmic signal. These latter results suggest that the *b*-specific N-terminal exon of human CD23 is able to actively retain the molecule at the cell surface.

The CD23a-specific exon is also a basolateral targeting signal

In mice, we previously showed that both CD23b and bΔ5 are targeted to the apical membrane of polarized MDCK cells (16), a steady-state localization in agreement with that of CD23 in enterocytes *in vivo* (26). In contrast, in humans, CD23 has been reported to be localized at both apical and basolateral membranes of intestinal epithelial cells (24).

To investigate the mechanisms responsible for such a difference, MDCK cells were stably transfected with the aLH and bLH constructs. The distribution of the chimeras in polarized cells was analyzed by confocal microscopy using ZO-1 staining as a marker of tight junctions and, therefore, of cellular polarization. As shown in Fig. 11, the bLH chimera was exclusively found at the apical membrane (*A*), suggesting that human CD23b behaves similarly as

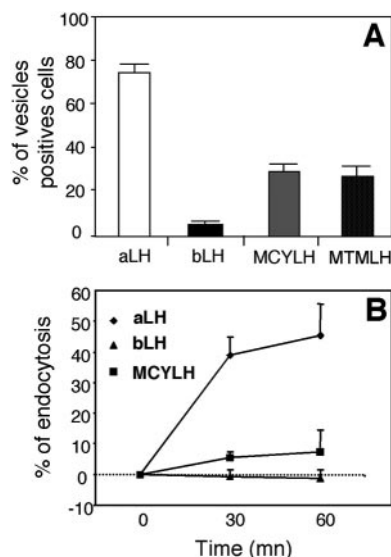


FIGURE 10. Roles of human CD23a- and CD23b-specific exons in endocytosis. *A*, HeLa cells transfected with plasmids encoding aLH, bLH, MCYLH, or MTMLH CD23 constructs were examined for B3B4 uptake by immunofluorescence as indicated in Fig. 2. The data (mean $\pm$ SE) presented in this figure are the means of at least three independent experiments. *B*, HeLa cells transfected with aLH, bLH, or MCYLH CD23 form-encoding plasmids were tested for PE-conjugated B3B4 uptake by flow cytometry as described in Fig. 4. The data (mean $\pm$ SE) presented in this figure are the values obtained from at least three independent experiments.

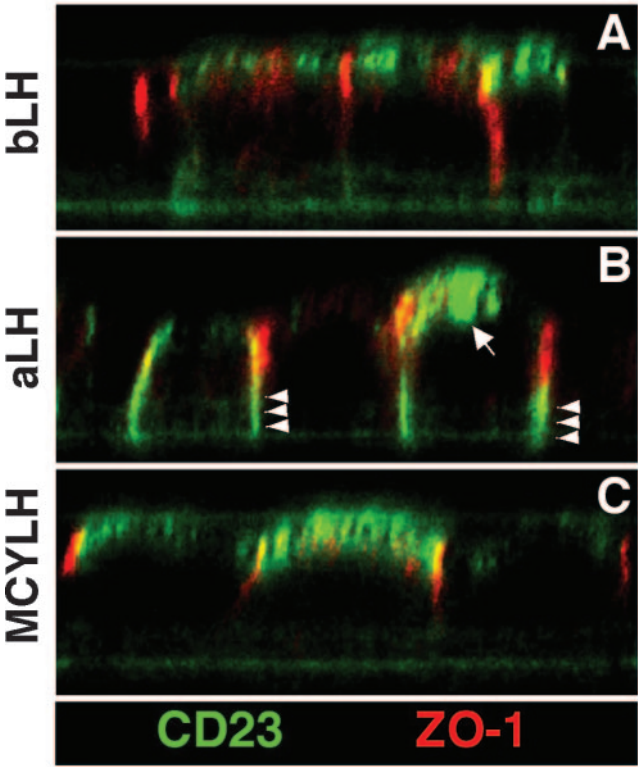


FIGURE 11. Human CD23a is targeted to the basolateral membrane of polarized MDCK cells. MDCK cell lines stably expressing bLH (A), aLH (B), or MCYLH (C) chimeras were grown for 6 days on Transwell filters, washed, fixed, permeabilized, and stained for CD23 (green) and ZO-1 (red). The samples were analyzed by confocal microscopy, and *y* and *z* sections are shown. The ZO-1 staining identifies the tight junctions and delineates the apical (*above*) and basolateral (*below*) membranes. Arrowheads show the basolateral staining observed for aLH. The arrow indicates apical vesicular structure decorated by aLH.

the murine form. Strikingly, aLH was found mainly at the basolateral membrane in the same conditions, as shown by the lateral CD23 staining observed below patches of ZO-1 (Fig. 11B, arrowhead). The aLH chimera was also found in bright vesicular structures in the apical region of the cells (Fig. 11B, arrow). This staining, which appeared clearly distinct from classical apical membrane staining in horizontal sections (data not shown), probably corresponds to the apical recycling endosomes (27).

Interestingly, tyrosine-based signals for clathrin-dependent endocytosis have also been implicated in the targeting of transmembrane proteins to the basolateral membrane of polarized epithelial cells (28). We tested whether the tyrosine-based signal characterized in the cytoplasmic tail of human CD23a could also be responsible for the presence of the CD23a chimera at the basolateral membrane of polarized MDCK cells. As expected, upon stable expression in MDCK cells, the MCYLH chimera, which lacks the CD23a-specific exon and therefore the critical tyrosine residue, was exclusively found at the apical membrane of polarized MDCK (Fig. 11C), showing a distribution very similar to that of bLH (Fig. 11A).

Discussion

The data presented in this manuscript provide important new data that allow a better understanding of the endocytic behavior of CD23 splice forms in both mice and humans and, thus, of their specific functions in both species. In addition, our results stress the strong divergence between the two species in the mechanisms involved in the control of endocytosis of CD23 splice forms. Finally,

in the more specific case of food allergies, our results show that in humans the expression pattern of CD23 splice forms in intestinal cells as well as their subcellular distribution in polarized cells is also strikingly different from those in mice.

The results presented in this study confirm and extend those initially obtained in the report by Yokota et al. (15), which represented the only true mechanistic study of CD23 endocytosis. In that study, human CD23a was found to mediate the internalization of membrane-bound Abs and was suggested to be taken up by the clathrin-dependant pathway based on its localization in CCPs by electron microscopy. These data were confirmed in our study, and the use of siRNA against the AP-2 complex directly shows that CD23a is effectively internalized through CCPs (Fig. 6). In addition, the localization of CD23a in endosomes at steady state indicates that its internalization is a constitutive process (not induced by prebinding of Abs; Fig. 9). The determinant responsible for this constitutive clathrin-dependent endocytosis was mapped in the CD23a-specific exon, a result in agreement with previous data that indirectly suggested that tyrosine 6 may be part of this signal. This tyrosine residue is found in a Yxx ϕ context (YSEI, Fig. 12), in which ϕ represents an hydrophobic residue (F, I, L, M, or V) and therefore fits with classical, tyrosine-based, clathrin-dependent internalization signals (29). This signal seems to be the only one present in the cytoplasmic tail of CD23, because no difference in

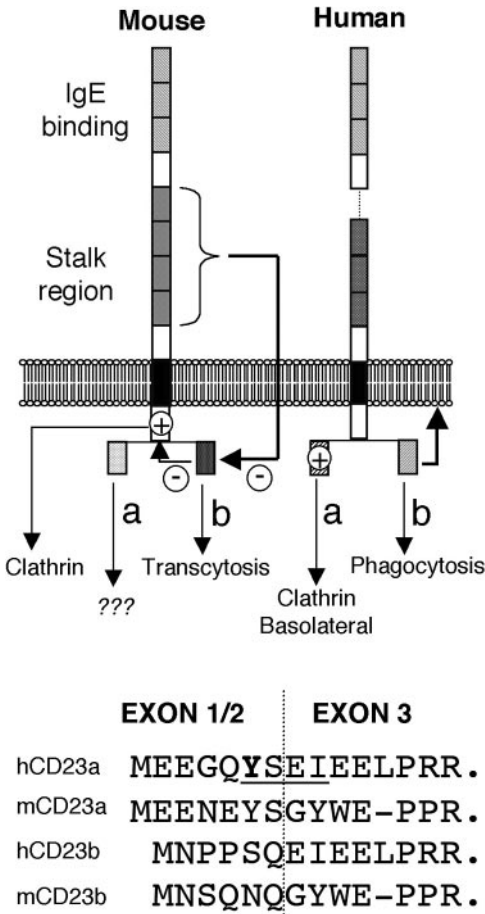


FIGURE 12. Structural and functional comparisons of mouse and human CD23. *Upper panel*, Monomers of mouse and human CD23 proteins. Specific endocytic signals, regulatory elements, and functions are indicated for each CD23 subdomain studied in this figure or elsewhere. *Lower panel*, Alignment of both human and mouse N-terminal CD23a and CD23b sequences. The putative clathrin-dependent endocytic signal in human CD23a is underlined.

internalization efficiency could be observed between a mutant lacking this signal only and a mutant lacking all of the cytoplasmic tail (Figs. 9 and 10).

Our results also show that murine CD23 contains determinants for constitutive internalization through CCPs (Fig. 6). However, the mechanisms involved in the control of CD23 endocytosis are completely different. First, in the murine model, determinants for clathrin-dependent endocytosis were localized not in the CD23a-specific exon, but, rather, in the region of the cytoplasmic tail shared by all the CD23 splice forms (Figs. 3–5). The exact function of CD23a-specific exon in mice remains to be determined (Fig. 12). Second, we could not map any classical endocytic motif. This region does contain a tyrosine residue, but it is not present in a consensus internalization signal (NPxY or Yxx ϕ), and its mutation to alanine does not show any effect on endocytosis (data not shown). Finally, the mechanisms responsible for the differential endocytosis of CD23a vs CD23b appear to be completely different, because the endocytic behavior of murine CD23 is not due only to the presence or the absence of a given endocytic signal. Instead, our results suggest that the CD23b-specific exon negatively regulates the signal for endocytosis. Indeed, although the classical CD23b is not internalized, the MCY mutant in which the CD23b-specific exon has been removed is efficiently internalized through CCPs (Figs. 3–5). In addition, the modulatory function of the CD23b-specific exon is under control of the extracellular stalk region, because deletions in this domain allow internalization of CD23 molecules even in the presence of CD23b-type intracytoplasmic region (Fig. 12). It does not seem that this function of the stalk domain is mediated by a specific exon, because deletion of exon 5, 6, or 7 alone or in combination appears to result in the constitutive clathrin-dependent endocytosis of the resulting CD23 molecule (Fig. 2) (8, 16).

The exact mechanisms allowing control of the intracytoplasmic *b*-specific exon by the extracellular stalk region is not easy to understand. However, it may be related to the outside-in signaling characterized for integrins, in which modification of the extracellular domain, such as ligand binding, acts on the intracellular part of the molecule. Recent studies using fluorescence resonance energy transfer suggested that outside-in signaling indeed results in separation of the cytoplasmic domains of α - and β -chains (30). Similarly, in the case of CD23, we can hypothesize that deletion of exons of the extracellular stalk domain may modulate the orientation and/or accessibility of the *b*-specific exon to specific cytoplasmic partners. Such possible partners remained to be characterized; however, examples of receptors actively retained at the plasma membrane through interaction with actin-based cytoskeletal elements such as filamin have already been described (31, 32). The indirect interaction of the *b*-specific exon with actin is also in agreement with the specific involvement of human CD23b in phagocytosis of IgE-opsonized particles (15) and in the apical to basolateral transcytosis of IgE/allergen complexes in polarized epithelial cells (16), two processes dependent on the actin-based cytoskeleton (33, 34). In addition, human *b*-specific exon is involved in the active retention of CD23b at the plasma membrane (Fig. 10), suggesting that at least this function is conserved in both humans and mice.

Together, our previous reports and the results presented in this study show that functions of murine CD23b are tightly regulated by alternative splice events within the stalk domain regulating its endocytic properties. However, our most recent study indicates that the *b* Δ 5 splice form is probably the only relevant one, because it is the only one that shows high affinity binding for IgE and is effectively expressed in intestinal cells *in vivo* (16). In addition, the fact that *b* Δ 5 is involved in the transepithelial transport of free IgE

(16) stresses that this splice form is probably involved in specific functions *in vivo*. Interestingly, a rat *b* Δ 5-like splice form could be found in the databanks (accession no. X73579.1), suggesting that alternative splicing of the stalk domain is a conserved process among rodents. Finally, a very recent study implicated mutations in CD23 in the hyper-IgE response in NZB mice (35). Interestingly, mutations were localized in the IgE binding domain as well as in exon 5. The relative contributions of these mutations in the phenotype were not investigated, but these results stress the crucial role of exon 5 in the regulation of CD23.

The fact that the stalk domain undergoes tight regulation by alternative splicing in mice, but not in humans, encourages us to take a more careful look at the structural differences in this domain between the two species. As previously reported (2), it is interesting to notice that the human stalk domain is shorter than that in the murine molecule. Direct comparison of mouse exons 5–8 with human exons 5–7 by sequence alignment indicates that the presence of an additional exon in mice is probably due to duplication of exon 7 in this species (data not shown). Indeed, exons 5–7 are well conserved across species, and the additional murine exon 8 shows the strongest homology with exon 7. Thus, it seems that as the human stalk region already lacks one exon, it does not need further regulation by alternative splicing.

In conclusion, divergent strategies have been found in humans and mice to control and regulate the expression and functions of CD23 splice forms. In the specific case of intestinal allergy, our results show that mouse intestinal cells express both CD23b and *b* Δ 5 (8, 9, 16). The difference in endocytic behavior between these two splice forms was correlated with different functions *in vivo* being implicated, respectively, in apical to basolateral transport of IgE/allergen complexes and of free IgE (16). In humans, intestinal cells coexpress CD23a and CD23b (Fig. 7), and we could not detect the expression of *b* Δ 5-like splice forms. Interestingly, CD23a and CD23b presented opposite distributions in polarized cells, with CD23a being found at the basolateral membrane, whereas CD23b was mainly apical (Fig. 11). These results are in agreement with those obtained by immunocytochemistry of human biopsies, showing that CD23 was found on both membranes of enterocytes (24, 36). Indeed, the coexpression of both CD23 splice forms in polarized human intestinal cells is likely to result in such a steady-state staining. The situation in humans is therefore again strikingly different from that in mice, because we could not find any of the CD23 splice forms, even CD23a, at the basolateral membrane of polarized epithelial cells (16) (data not shown). Thus, although CD23b is likely to exert conserved functions at the apical membrane of enterocytes, it is tempting to speculate that CD23a in humans may functionally replace *b* Δ 5 in transporting free IgE.

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Disclosures

The authors have no financial conflict of interest.

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