

Differential role for CD23 splice forms in apical to basolateral transcytosis of IgE/allergen complexes.

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Running title: transcytosis of IgE/allergen complexes by CD23

Key words: allergy, IgE, CD23, endocytosis, clathrin, transcytosis.

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Summary

The low affinity receptor for IgE (CD23) was recently implicated in the trans-epithelial transport of IgE-allergen complexes from the luminal side of enterocytes in animal models for intestinal allergy. Here, the respective functions of CD23 splice forms, *b* and *bD5*, in this apical to basolateral transport event have been investigated. First, the new *bD5* splice form was further characterized providing evidences that it binds IgE with high affinity, that its expression is induced by sensitization, and that *bD5*, unlike the classical *b*, undergoes constitutive internalization through clathrin-coated pits. These results suggested that the two CD23 splice forms were likely involved in different transcytotic events. MDCK cell lines expressing either *b* or *bD5* were generated to directly test this hypothesis. In both cell lines, CD23 splice forms were localized at the apical membrane as in enterocytes from sensitized mice. Using mouse monoclonal IgE, we obtained evidence showing that *bD5* mediates the apical to basolateral transport of free IgE whereas classical *b* is much more efficient in mediating the transcytosis of IgE/allergen complexes. Altogether, the present results shed new light on the role of CD23 species in IgE/allergen transepithelial transport and provide a new powerful physiological tool to study apical to basolateral transcytosis, a process which remains poorly characterized.

Introduction

In the intestine (and possibly the lung) of sensitized rodents, the rapid and specific transport of the sensitizing antigen (allergen) from the mucosal (apical) to the serosal (basal) side of the epithelium appear to be mediated by CD23, the low affinity receptor for IgE (1-4). In current models, allergen-IgE complexes present in the lumen bind to CD23 and this mediates their internalization from the apical membrane. Transcellular transport then proceeds via an as yet uncharacterized transcytotic pathway to the serosal milieu. The presence of allergens in the sub-epithelial compartment subsequently leads to the activation of resident mast cells which release pro-inflammatory mediators responsible for classical anaphylactic symptoms observed in allergic patients (5).

CD23 (RFcεRII) is fundamentally different from the high-affinity IgE receptor (RFcεRI) expressed by mast cells, since it corresponds to a single type II transmembrane glycoprotein composed of a N-terminal short cytoplasmic tail, a single transmembrane domain, a coil-coiled domain (also called stalk region) responsible for high-affinity IgE binding and the C-terminal lectin-like IgE binding domain (6-11). It also differs from other classical low affinity Immunoglobulin Fc-receptors since it binds monomeric IgE with reasonably high affinity (12). Two major CD23 splice forms (*a* and *b*), generated by the use of alternative transcription initiation sites, have been described both in mice and in humans (13-15). 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*) (see Figure 2 for details). CD23a is mainly expressed on B-lymphocytes where it is believed to play a role in the internalization of IgE-bound antigens resulting in their subsequent processing and MHC-II restricted presentation (16, 17). Indeed, human CD23a is constitutively internalized through clathrin-coated pits (18). The expression of CD23b is induced in several cell types including B-lymphocytes, macrophages and keratinocytes by interleukin-4 and/or lipopolysaccharide (15, 19, 20). Human CD23b is not constitutively internalized (18). However, it has been implicated in the phagocytosis of IgE-opsonized particles when expressed in macrophages (18). Its specific role in non-immune cells has not been clearly defined.

In mice, until recently, only the *a* splice form was clearly described, and the existence of the CD23b remained controversial (6). However, our previous studies established that mouse intestinal epithelial cells (*in vivo* and freshly isolated) and cell lines (IEC-4) do express CD23b (3, 21), suggesting that the *b* splice form might be involved in IgE-allergen transcytosis across the epithelium. Moreover, a systematic sequencing of CD23 RT-PCR products identified two novel CD23b-derived alternative splice forms lacking either exon 5 (*bD5*) or exon 6 (*bD6*) in the extracellular stalk region. These two new splice forms were potentially interesting since, in contrast with classical CD23b, they showed efficient endocytosis being able to internalize receptor bound anti-CD23 antibodies (3).

This study was designed to directly show

the requirement of CD23 expression in the apical to basolateral transport of IgE/allergen complexes and to better understand the respective role of CD23 splice forms in this process. With this goal in mind, we first analyzed IgE binding properties of the newly identified splice forms and showed that only *bD5* is a functional high-affinity IgE receptor. The expression pattern and endocytic functions of this splice form were further characterized. Finally, the respective function of CD23b splice forms in transcytosis of IgE or IgE/allergen was directly investigated in stable MDCK cell lines expressing classical *b* or *bD5* murine CD23 splice forms.

Results

The *bD5* splice form is a functional IgE receptor

Murine CD23 binds IgE with either a low or high affinity depending on the stalk region. Both exons 5 and 6, which are deleted in the new CD23b splice forms *bD5* and *bD6*, respectively, are part of the stalk region and exon 6 was shown to be required for high affinity IgE binding (12). In contrast, the direct role of exon 5 in this process was never investigated and it was assumed that it was likely to exert similar functions due to its sequence homology with the other exons of this domain (exons 6-8). Altogether, these data suggested that the new identified splice forms were unlikely to be high affinity IgE receptors. We previously tested the IgE binding properties of *bD5* and *bD6* splice forms by fluorescence microscopy and reported that they did both bind and internalize free IgE (3). However, trying to reproduce these results recently we were unable to detect any IgE binding for *bD6* (data not shown). To be definitively assess the IgE binding properties of the CD23b splice forms, a classical Scatchard analysis was performed on CHO cells transiently transfected with *b*, *bD5* and *bD6* expressing plasmids. The results obtained with this technique were consistent with our more recent results and confirmed that only *bD5* did bind IgE with a similar biphasic affinity as classical *b* splice form (Figure 1 and not shown). These results confirmed the role of stalk region and exon 6 in high affinity IgE binding (12). They also show the unique role of exon 5 in the stalk region being homologous to the other exons of this region in sequence but not in function.

Expression of *bD5* is induced by sensitization *in vivo* and by Interleukin-4 *in vitro*

The results presented above clearly indicate that the *bD5* splice form is likely involved in physiological functions *in vivo*. This hypothesis is in agreement with our observations that *bD5* transcripts are more abundant than *bD6* in intestinal cells ((3) and data not shown). Our previous results also established that intestinal cells from sensitized animals express CD23b but not CD23a (3), however the primers used in that studies were designed to amplify the full length of CD23b and therefore did not allow the direct detection of *bD5*. Two new primers pairs were then designed to amplify CD23 species. The first pair was designed to amplify shorter fragments of CD23

including exons 2 to 6 to shorten the size of PCR products and therefore improve the efficiency of the amplification reaction. The second utilize the same upstream primer in combination with a downstream primer specific for the *bD5* splice variant (see Figure 2 for details). Preliminary tests revealed that the latter primers did preferentially amplify the *bD5* splice form from a mix of *bD5* and CD23b encoding plasmids (data not shown). These two new primers pairs were then used to amplify CD23 by RT-PCR from jejunum fragments of control mice or mice sensitized with horseradish peroxidase. The homogeneity of the samples was first controlled amplifying the ubiquitous ribosomal RNA 18S and the observation that bands of similar intensities were obtained from all samples (Figure 3, C) indicated that the cDNAs used were indeed comparable CD23 expression in the intestine of sensitized animals as well as in control animals was confirmed (Figure 3, A) and appeared to be up-regulated upon sensitization (compare lanes 1 and 2 to lanes 3, 4 and 5), in keeping with our previous observations (3, 21). This increased expression of CD23 in sensitized animals correlated with the expression of the *bD5* splice form that was only detected in sensitized animals (Figure 3, B, lanes 3, 4 and 5). Similar results were obtained using the intestinal epithelial cell line IEC-4 in which the expression of *bD5* was induced by interleukin-4 treatment (Figure 3, D-E). Indeed, interleukine-4 plays a central role in allergy and was shown to induce the expression of CD23b in B lymphocytes as well as in various other cell types (15). Altogether, these results show that *bD5* is effectively expressed *in vivo*, at least following sensitization, and therefore suggest its possible role in the allergic reaction.

bD5 is constitutively internalized

We previously showed, using a fluorescence microscopy-based approach, that in contrast to the classical *b* splice form *bD5* is efficiently internalized (3). Indeed, *bD5* expressing cells efficiently internalized membrane bound anti-CD23 antibody or free IgE (Figure 4 and (3)) as shown by the extensive colocalization of anti-CD23 staining with internalized transferrin (Figure 4, C and D). In the same conditions, CD23b remained mainly on the plasma membrane (Figure 4, A and B).

These results suggested that the *bD5* splice form was constitutively internalized. However, we could not exclude the possibility that the observed endocytosis of *bD5* was an induced phenomenon due to the use of pre-bound antibodies to follow CD23 internalization. To definitively rule out this possibility, we checked the intracellular localization of CD23 splice form at steady state. Indeed, if the *bD5* splice form undergoes constitutive internalization, it was expected to be localized in endosomes in the absence of any added extracellular ligand. To test this hypothesis, the intracellular localization of *bD5* at steady state was then compared to that of several early/sorting endosomal markers such as internalized transferrin and green fluorescent protein (GFP)-tagged Hrs (22) and 2xFYVE (23) constructs (data not shown and Figure 4, E and F). As noted in our previous study (3), *bD5* staining was observed on the

plasma membrane as well as in numerous intracellular vesicular structures (Figure 4, E and data not shown). This intracellular vesicular staining showed obvious colocalization with the GFP-2xFYVE construct (Figure 4, F), which stains the overall early/sorting endosomal compartment (23). These results show that the efficiently internalized *bD5* splice form is present in endosomes at steady state, strongly suggesting that it undergoes constitutive internalization.

Set up of a flow-cytometry based assay to quantify CD23 internalization

The protocol used above and in our previous study to analyze the endocytosis of CD23 splice forms was useful in analyzing the basic internalization properties of CD23 splice forms. However, it could be very difficult to use this assay to determine the effects of inhibitors of endocytic pathways, i.e. expression of mutants or drugs, considering the effects could be subtle especially in the face of issues such as cell-to-cell variability. Therefore, we developed a new fluorescence-based endocytosis assay in which the intracellular accumulation of anti-CD23 antibodies was directly quantified by flow cytometry (see material and methods and Figure 5 for details). In this assay, the internalization of CD23 was quantified as the intracellular accumulation of membrane bound phyco-erythrin (PE)-conjugated anti-CD23 antibodies which would then become resistant to extracellular trypsin (Figure 5, A). Importantly, the same anti-CD23 antibody (B3B4) was used in the two different endocytosis assays. The endocytic properties of CD23 splice forms were then re-investigated and, as shown in Figure 5, the results obtained by this new assay were very similar to those obtained with the previous test; the *bD5* splice form was internalized 2.5 to 3 times more efficiently than CD23b (Figure 5, B). In addition, the observed internalization for CD23b is likely to correspond to background level since it is similar to what observed for a CD23 mutant lacking the intracytoplasmic region (data not shown).

***bD5* is internalized through clathrin-coated pits**

We next used the flow-cytometry based assay to elucidate the mechanisms underlying the constitutive internalization of *bD5*. It has been shown that human CD23a and CD23b displayed different endocytic properties. The molecular determinants responsible for that differential trafficking were not completely characterized but a clathrin-dependent endocytic signal was shown to reside only in the a specific exon of human CD23. The absence of endocytosis of CD23b was then explained by the absence of a encoding exon. In contrast, the *b* splice forms was involved in IgE-dependent phagocytosis when expressed in macrophages (18). The mechanisms involved in the endocytosis of murine CD23 splice forms were never investigated and it is assumed that they behave as their human counterparts.

The simplest hypothesis to explain the constitutive endocytosis of the *bD5* splice form is that it undergoes internalization through clathrin-coated pits. To directly test this hypothesis we took advantage of recent studies showing that siRNA targeting subunits of the AP-2 complex could be

successfully used to inhibit the formation of most if not all clathrin-coated pits and therefore to specifically inhibit clathrin mediated endocytosis (24-26). Indeed, treatment of HeLa cells with siRNA against the μ 2 subunit of AP-2 (24) drastically reduced the cellular amounts of AP-2 complexes as evidenced by western-blotting experiments (data not shown) and by immunofluorescence using antibodies directed against the α -adaptin subunit (Figure 6, D). The latter experiments also stressed the dramatic reduction of clathrin-coated pits as evidenced by the almost complete loss of plasma membrane associated punctate staining for AP-2 compared to control luciferase siRNA treated cells (Figure 6, B and D, insets). The effect of AP-2 down-modulation on CD23 internalization was first tested by immunofluorescence. As in non treated cells, the *bD5* splice form was efficiently internalized in cells treated with control luciferase siRNA as shown by the vesicular endosomal staining observed for anti-CD23 antibodies after 30 minutes (Figure 6, A, arrows). In contrast, in AP-2 knock-down cells, the anti-CD23 antibodies remained on the plasma membrane (Fig. 6C), suggesting that internalization of *bD5* was greatly impaired.

The effect of AP-2 knock-down on internalization of *bD5* was then directly quantified using the flow-cytometry based assay. The results clearly show that the internalization of *bD5* was 2.5 times less efficient in AP-2 knock-down cells compared to luciferase siRNA mock-treated cells (Figure 6, E) and then reached levels similar to that observed for CD23b or for a CD23 construct lacking intracytoplasmic region (data not shown). In addition, a similar 60% inhibition was also observed for CD23a used as positive control for clathrin-dependent endocytosis, suggesting that it corresponds to the maximal inhibition observable for CD23.

Altogether these results show that *bD5* undergoes a constitutive internalization through clathrin-coated pits.

CD23b and *bD5* are found at the apical membrane of polarized MDCK cells

As the two CD23 splice forms expressed in intestinal cells, i.e. classical *b* and *bD5*, exhibit completely different endocytic properties, we therefore decided to analyze the consequences of these differences in their trafficking in polarized epithelial cells. We choose to express murine CD23 splice forms in MDCK cells, the most widely used model to study intracellular trafficking in polarized cells. This cell line presents several advantages including rapid growth, ease of transfection and the potential for polarization, particularly when grown on semi-permeable supports. In addition, in our specific system, MDCK cells are of canine origin and therefore neither express any cross-reacting IgE receptor, as shown by the absence of staining with anti-murine CD23 antibodies (Figure 7, A), nor bind murine IgE (data not shown and Figure 9). Stable MDCK cell lines expressing either *bD5* or CD23b were then generated by selection of individual CD23 expressing clones (see material and methods for details). Two cell lines expressing similar levels of cell surface CD23 splice forms (Figure 7, A) were further studied in detail.

The apical versus basolateral distribution of CD23 splice forms was analyzed by confocal microscopy. MDCK cells were grown on Transwell filters for 6 days and then stained simultaneously from basolateral and apical milieu with anti-CD23 antibody B3B4 (data not shown) or with monoclonal IgE (Figure 7) at 4°C. The cells on filters were then fixed, permeabilized and stained for ZO-1 as marker of polarization. The distribution of both classical *b* and *bd5* was very similar and mostly apical as visualized in horizontal cuts of the apical ZO-1-positive region of the cells (Figure 7, B), as well as in vertical sections showing CD23 staining associated with microvilli (Figure 7, C). Similar apical distribution of CD23 splice forms was also observed in cell lines corresponding to pooled CD23 expressing MDCK clones showing that this distribution is not clone specific (data not shown). In addition, we took advantage of the fact that CD23 is constitutively cleaved from the cell surface generating soluble form of the molecules (sCD23) corresponding to almost all the extracellular domain (27). We therefore analyze basolateral and apical secretion of sCD23 species by Western-Blotting using the B3B4 antibody which recognizes the IgE binding domain (28). As shown in Figure 8, sCD23 species were only found in apical milieu from both classical *b* and *bd5* MDCK cell lines, confirming that both splice forms are localized at the apical membrane of polarized cells.

CD23 splice forms are involved in different transepithelial transport events.

The MDCK cell lines were then used to test the role of each CD23 splice form in transcytosis of IgE and/or IgE/allergen complexes. The murine system allowed us to use a monoclonal IgE specific for dinitrophenyl (DNP) and DNP-coupled serum albumin (DNP-BSA) as model of allergen, a combination already used to study the internalization of IgE/allergen complexes by CD23b splice forms (3).

The transcytosis of free IgE from apical to basolateral media was first tested. MDCK cell lines were grown for six days on transwell filters then washed on both sides and IgE were introduced in the apical media all along the transport process, i.e. one hour at 37°C. This protocol was chosen because it is likely to reflect the physiological conditions in which IgE are found in the intestinal lumen (29). Basolateral media from 4 filters were then collected, concentrated and analyzed by western-blot using an anti-IgE antibody. In these conditions, the *bd5* splice form was much more efficient than classical *b* to transcytose free IgE (Figure 9, A). This result correlates with the data obtained in non-polarized cells showing that only *bd5* is able to internalize free IgE (3).

A modified version of the above protocol was used to analyze transcytosis of IgE/allergen complexes. In this modified protocol, we also tried to mimic the physiological situation in which the allergen are introduced in the intestinal lumen in which IgE are already present. Then, DNP-BSA was introduced in the IgE-containing apical milieu at the beginning of the incubation and for one hour. As above, the basolateral media from 4 filters were collected, concentrated and the presence of transported IgE in the basolateral milieu was

monitored by western-blotting. In parallel, in an attempt to check the integrity of the monolayer during the transport event, the same filters were washed processed for fluorescence microscopy to follow IgE and ZO-1 as indicated above. Surprisingly, in the presence of allergen, the two CD23 splice forms behaved in a complete opposite manner. Transported IgE present in the basolateral media were much more abundant for the *b* expressing cells than for those expressing *bd5* (Figure 9, B). This more efficient transport was also clearly observed by immunofluorescence (Figure 9, C). Indeed, numerous large IgE positive vesicles were observed in *b* expressing cells with increased staining also present along the basolateral membrane. In contrast, in *bd5* expressing cells, fewer vesicles were observed (Figure 9, D) and the IgE staining remained mainly in the apical region (not shown).

The correct ZO-1 staining in all the filters (not shown) indicated that the monolayers were indeed efficiently polarized. Together with the fact that IgE were not transported in cells that do not express CD23 and the differential transport efficiencies for splice variants (Figure 9) exclude the possibility that the transcytosis of IgE was due to non specific paracellular transport but indeed dependent on the expression of CD23.

DISCUSSION

The results presented in this study provide important new findings regarding the functional characterization of the new CD23 splice form *bd5* and shed new lights on the respective functions of CD23 splice forms in the transepithelial transport of IgE and IgE/allergen complexes.

***bd5*: unique role of exon5**

Among the new splice forms that we identified in our previous study (3), our present results show however that only *bd5* is likely to exert important function *in vivo* as suggested by its high affinity IgE binding (Figure 1). This hypothesis is strengthened by the fact that *bd5* transcripts were also found to be much more abundant than *bd6* (3) and by the fact that their expression was indeed induced by sensitization *in vivo* and by IL-4 *in vitro* (Figure 3). It is however important to notice that the classical *b* splice form seems to be predominantly expressed in intestinal cells (Figure 3) as suggested by our previous results (3).

The *bd5* splice form only differs from the classical *b* by the lack of exon 5 (Figure 2), which then appears to play a very important role in the regulation of CD23 functions. Indeed, the absence of exon 5 allows constitutive internalization of the resulting CD23b molecule (Figures 4, 5 and 6). This result was quite surprising since the *bd5* and classical *b* forms share exactly the same intracytoplasmic region which is likely to bear endocytic signals (G.M. and A.B., manuscript in preparation). These results suggest that exon 5 negatively regulates endocytosis of CD23b, its deletion allowing constitutive endocytosis. The

mechanisms involved in such regulation remains however poorly understood.

The first hypothesis we made was that exon 5 is required for oligomerization as suggested previously (12) and in consequences, that oligomerization may play important role in regulating the endocytosis of CD23b. However, the presence of oligomeric soluble forms of CD23 in apical media of MDCK expressing *bd5* (Figure 8) suggests that exon 5 is not required for oligomerization of the extracellular domain. Another hypothesis was that exon 5 is responsible for the interaction of CD23 with a putative plasma-membrane associated negative regulator which actively retains CD23b at the cell surface. However, this hypothesis is likely unsatisfactory since if such factor exists, it should also prevent endocytosis of exon 5 containing classical *a* splice form. The fact that classical *a* is efficiently internalized when expressed in the same cells as classical *b* argues against this hypothesis.

Even if the mechanisms involved in exon 5-mediated regulation are not easy to understand, the fact that exon 5 is not required for high affinity IgE binding (Figure 1) suggests its unique role within the stalk region since all the other exons of this domain are required for efficient IgE binding ((12), data not shown). Interestingly, murine and human CD23 splice forms differ by the presence of an additional exon within the stalk domain in the mouse molecule. Due to the high degree of homology of the exons constituting this domain (5 to 8 in murine CD23), it is difficult to clearly say if it is exon 5, which is effectively lacking in human CD23. However, these observations suggest that the mechanisms regulating CD23 functions in mice and humans are likely to be different.

As indicated above, the constitutive internalization of *bd5* through clathrin-coated pits suggests the presence of endocytic motif in its intracytoplasmic region and therefore in the intracytoplasmic region of classical *b* as well (Figure 2). This was not expected since it was assumed from studies on human CD23 that CD23b lacks any endocytic signal that is only present in the exon specific of the *a* splice form (18). Our unpublished observations suggest however that the cytoplasmic region of murine CD23b is required for clathrin-dependent internalization of *bd5*. Together with the data showing that murine CD23a is internalized through clathrin-coated pits (Figure 6), these results suggest that the endocytic signals are likely present in the cytoplasmic region shared by all CD23 splice forms. However we could not map classical highly efficient Tyrosine or di-Leucine based endocytic signals (30) in this region (G.M. and A.B., manuscript in preparation). This is likely to explain the low efficiency internalization observed for CD23 molecules. Interestingly, the internalization rate observed for *bd5* as well as for classical *a* splice form in AP-2 knock-down cells reach the ones observed for mutant forms lacking the cytoplasmic region (data not shown). The latter results suggest that the observed 60% inhibition observed for both *bd5* and classical *a* splice forms (Figure 6) represents the maximal effect that could be observed for CD23. These results again argue that human and mice CD23 behave differently.

CD23 in polarized epithelial cells

The generation of MDCK cell lines stably expressing classical *b* and *bd5* allowed us for the first time to dissect the respective functions of these splice forms in IgE/allergen transcytosis and also to definitively show that expression of CD23 is indeed directly required for this process.

Our data show that both CD23 splice forms were found to reside at the apical membrane of MDCK cells (Figures 7 and 8). These results are in agreement with those obtained *in vivo* showing that in mice and rats models of intestinal allergy, CD23 was mainly found on the apical membrane of enterocytes associated with microvilli (1, 2, 21). They are however in contrast with data obtained in human patients in which CD23 molecules have been found on both apical and basolateral membranes of enterocytes. These results suggest again that human and rodent CD23 are differentially regulated and may exert different functions.

The most exciting of our results is that classical *b* and *bd5* splice forms are involved in different transport events from the luminal side of epithelial cells to the basolateral milieu; *bd5* being involved in the transport of free IgE whereas IgE transport of CD23b only occurs in the presence of allergen. Interestingly, in both cases, the transported IgE remained intact as shown by the presence of only one band of the expected size for intact IgE in western-blot (Figure 9). These observations are in agreement with our most recent study showing that the allergen was "protected" from degradation during CD23-dependent transport across intestinal biopsies of sensitized mice (21). Altogether, these results are in agreement with the proposed model for allergen transport in allergic reaction (see introduction) and suggest that the CD23-mediated transport allow the translocation of intact IgE/allergen complexes that are delivered in the native conformation to the serosal immune system.

These differences in IgE and/or IgE/allergen transport efficiencies between the CD23 splice forms correlate with their respective endocytic properties in non-polarized cells. The results concerning the transport of free IgE by *bd5* are in agreement with the data obtained showing its constitutive internalization (Figures 4 to 6, and (3)) and its capacity to internalize free IgE (3). The highly increased capacity of classical *b* to transport IgE in the presence of allergen is also in agreement with our previous observations indicating that it is able to internalize IgE only in the presence of allergen (3). Interestingly, expression of classical *b* has been first described in macrophages in which it was implicated in the phagocytosis of IgE-opsonized particles (18). These two processes require the aggregation of CD23 molecules by multivalent IgE that may induce a specific endocytic pathway at the apical membrane of epithelial cells.

The physiological function of apical to basolateral transport of free IgE by *bd5* is may be related to clearance/recycling of luminal IgE (29) or to the transfer of maternal IgE which appears to occur through the gastrointestinal tract of the fetus (31). In addition, the transport of free IgE by *bd5* also suggests its possible role in the transport of

monovalent allergens, which do not induce the cross-linking of CD23-bound IgE and are then likely to behave like free IgE.

Our first hypothesis was that *bd5* would be involved in basolateral to apical transport of IgE. Indeed, IgE are produced by plasma cells, which are found in the local gut-associated immune system and/or are coming from blood and have to be transported across the epithelial cells to the lumen to exert their functions. Indeed, such an active transport was characterized *in vivo* (32). However, we could not detect any specific basolateral binding of IgE (Figure 7 and data not shown) and our efforts to observe basolateral to apical IgE transport remained unsuccessful (data not shown). Therefore, the mechanisms responsible for as well as the possible role of CD23 in this process remain to be determined. Interestingly, CD23 is found on both apical and basolateral membranes in human patients suggesting a possible role of CD23 in basolateral uptake of free IgE. This stresses again that the functions played by CD23 function in allergen transport may show important differences between species.

CD23 and apical endocytosis:

Most of our knowledge of transcytotic pathways in polarized epithelial cells was acquired by the characterization of the intracellular trafficking of immunoglobulin (Ig) receptors. Indeed, the polymeric Ig receptor (pIgR) enables the characterization of the basolateral to apical transport pathway and more recently the neonates Ig receptor (FcRn) was used as a possible model system of apical to basolateral transport (33). Our results indicate that CD23 appears to be a new powerful physiological model to characterize both the apical to basolateral transcytosis pathway and of course apical internalization. Indeed, the mechanisms underlying clathrin-coated vesicle formation at the apical membrane were shown to be different than at the basolateral membrane (34-36). Therefore, *bd5* could be used as a physiological marker to follow apical clathrin-dependent endocytosis whereas classical *b* splice form may represent a unique marker of a new inducible apical endocytosis pathway that remains to be characterized.

Materials and Methods

Animals and sensitization

Female Balb/C mice, aged 5-6 weeks (Charles River, Saint-Aubin les Elboeuf, France) were used in this study. Mice were actively sensitized by intraperitoneal injection of 100 µg horseradish peroxidase (HRP type VI; Sigma, St Louis, MO, USA) (HRP-sensitized group, n=3) in 0.2 ml alum, together with 0.1 ml (50 ng) of pertussis toxin (Sigma) as adjuvant. Control mice were injected with phosphate buffer saline (PBS) and alum. The animals were sacrificed on day 14-15 after sensitization when the jejunum was removed and treated as indicated below. All animal experiments were conducted with the approval from the Necker University Animal Care Committee.

RNA extraction and RT-PCR

Reverse transcription-polymerase chain reactions (RT-PCR) were performed on total RNA extracted from mouse jejunal segments or IEC-4 cells using the RNeasy Mini kit (Qiagen, Courtaboeuf, France). Segments of jejunum from control or sensitized BALB/c mice were washed in phosphate-buffered saline (PBS) and cut into 3 mm pieces for RNA extraction. Genomic DNA contamination was removed from RNA samples with DNA free kit (Ambion, Austin, TX, USA). Purified RNA was analyzed by electrophoresis to check its integrity and quantified by measuring absorbance at 260 nm. Total RNA (2 µg) was reverse-transcribed with both Oligo d(T)16 and random primers. Briefly, RNA, Oligo d(T) (0.5 mg) and random primers (0.15 mg) were incubated at 70°C for 10 minutes then at 4°C for 5 minutes. A mixture containing 200 units M-MLV, 0.5 mM dNTP and 10 mM DTT (Invitrogen, Carlsbad, CA, USA) was then added and samples were incubated at 42°C for 75 minutes. A final step at 70°C for 5 minutes was performed to inactivate the reverse transcriptase. PCR was then performed on 100 ng of cDNA from jejunum or from IEC-4 cells by adding a mix containing 1 unit Platinum [®]Taq High Fidelity (Invitrogen), 1x PCR buffer, 4 mM MgSO₄, dNTP (0.2mM each), 0.2 mM of each primer (CD23: upper 5'-CAG AAT GTG CTG CTG TGC AAG ACG TGG G-3' and lower 5'- CTT GCA AGT TTT GTG ACA TCT GAA CAA-3'; *bd5*, upper 5'- CAG AAT GTG CTG CTG TGC AAG ACG TGG G -3' and lower 5'- AAG TTT TGT GAC ATC TGA ACA ACA TTC-3'; ribosomal RNA 18S : upper 5'- CGG CTA CCA CAT CCA AGG AA-3' and lower 5'-GCT GGA ATT ACC GCG GCT-3'). The DNA thermal cycler (Gene Amp, PCR system 2700, Applied Biosystems, Foster City, CA, USA) was programmed to perform a protocol as follows: 94°C for 3 min for 1 cycle; 94°C for 1 min, 60°C for 1 min, and 68°C for 1 min for 40 cycles; and 68°C for 7 min for final extension. For each primer couple, control amplifications were performed in which cDNA was omitted from the PCR reactions. RT-PCR products were analyzed by electrophoresis (agarose 1.5%) using the 1Kb plus DNA Ladder molecular weight marker from Invitrogen.

DNA Constructs

Plasmids (pCR3.1, Invitrogen) encoding murine CD23 splice forms *b* and *bd5* were described previously (3). The CD23a splice form was transferred from pCDNA1 (12) into the pCR3.1 vector using HindIII and NotI restriction sites. The GFP-tagged 2xFYVE constructs was a kind gift from H. Stenmark (23).

Cell culture and Transfection

The mouse intestinal IEC-4 (37) and the human epithelial HeLa (ATCC, Manassas, VA, USA) and canine MDCK strain II (a kind gift of A. Zahraoui, Curie Institute, Paris, France) cell lines were grown in DMEM media supplemented with 10% foetal bovine serum, 20 mM L-glutamine, and 5 mg/ml streptomycin sulfate (Life Technologies, Invitrogen). Murine IL-4 was obtained from R&D systems. MDCK monolayers were obtained by seeding 1 million cells on a 12 mm diameter, 0.4 µm pore size Transwell

(Costar, Bethesda, MD, USA).

For transient transfections, HeLa cells were grown one day before transfection in six wells plates and directly on coverslips for fluorescence microscopy studies. Cells were then transfected with a maximum of 5 µg of plasmids per well using the calcium phosphate transfection kit from Invitrogen and were processed the following day. Stable MDCK cell lines were obtained by transient transfection of *b* or *bd5* encoding plasmids using the same protocol as for HeLa cells. Selection was started one day after transfection by adding 0,6 mg/ml Geneticin (Invitrogen).

For siRNA treatment, HeLa cells were transfected with siRNA duplex (Dharmacon, Lafayette, CO, USA) specific for human μ 2 subunit of AP-2 or luciferase (control) with Oligofectamine (Invitrogen) according to the manufacturer's instruction and to the protocol developed by Motley and colleagues (24) (μ 2 siRNA : 5'- AAG UGG AUG CCU UUC GGG UCA -3', Luciferase siRNA : 5'- CGU ACG CGG AAU ACUU CGA TT -3'). Briefly, 200 pmol of siRNA were transfected the first day. On the second day, cells were transfected with 5ug DNA, incubated for about 6 hours and then washed twice in PBS and again transfected with 200pmol siRNA. Cells were processed for B3B4 endocytosis on the third day.

Immunofluorescence

To characterize endosomal localization of CD23 splice forms at steady state, HeLa cells were transiently transfected with the *bd5* and GFP-2xYFVE encoding plasmids. The following day, cells were washed in 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. Cells were then washed and incubated in PBS supplemented with bovine serum albumin (BSA, Sigma) at 1mg/ml (PBS-BSA) with an Alexa594-conjugated goat anti-rat IgG secondary antibody (Molecular Probes, Invitrogen).

To control for AP-2 expression in siRNA treated cells, cells were fixed and incubated with a monoclonal mouse anti α -adaptin antibody (AP.6, ATCC) in permeabilization buffer. Cells were then washed and incubated in PBS-BSA with an Alexa594-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Invitrogen).

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, Germany) attached to a cooled CCD camera (Micromax, Princeton Instruments, Trenton ,NJ, USA). The pictures were taken using MetaMorph (Universal Imaging, Downingtown, PA, USA) and the final images were obtained using NIH image (<http://rsb.info.nih.gov/nih-image/>) and Photoshop (Adobe Systems Inc., San Jose, CA, USA).

CD23 internalization indicated by fluorescence microscopy

In this assay, the internalization of CD23 was characterized by fluorescence microscopy following the intracellular accumulation of plasma-membrane

bound anti- CD23 antibody (B3B4, rat monoclonal IgG2a, (28)). The endosomal localization of the internalized antibody was followed using transferrin as a marker of early endosomes as previously described (3). Briefly, HeLa cells transiently 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 one hour at 4°C in the presence of B3B4 (50 µg/ml) in PBS -BSA. Cells were then washed two times in DMEM-BSA (1 mg/ml) and incubated for 30 min at 37°C in DMEM-BSA containing 6 mg/ml Alexa594-conjugated transferrin (Molecular Probes, Invitrogen). The cells were rapidly cooled to 4°C using cold DMEM-BSA, washed in cold PBS and fixed. To reveal the internalized anti-CD23 antibody, cells were incubated for 30 minutes at room temperature in the presence of an Alexa488-conjugated goat anti-rat IgG secondary antibody (Molecular Probes, Invitrogen) in permeabilizing buffer (PBS-BSA, 0.1% triton).

CD23 internalization indicated by flow-cytometry

In this assay, the intracellular accumulation of membrane-bound phyco-erythrin (PE) conjugated B3B4 (Becton Dickinson, Franklin Lakes, NJ, USA) was quantified by flow-cytometry. HeLa cells grown in 6 wells plates were transiently transfected with CD23 encoding plasmids the day before. Cells were maintained adherent during the assay, incubations and washes being conducted in plates. Cells were washed once in cold PBS and incubated with B3B4-PE (1:100) for 1 hour at 4°C in PBS-BSA (10 mg/ml). After two washes in cold PBS-BSA, the cells were allowed to internalize for 0, 30 or 60 min at 37°C in DMEM-BSA (10 mg/ml) and then rapidly cooled with cold medium on ice. After two washes in cold PBS, the remaining plasma membrane associated B3B4-PE was cleaved by a four minutes 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 in each wells, and cells were resuspended by pipetting up and down. The cells were finally washed by centrifugation at 2500 rpm for 5 minutes at 4°C, resuspended in cold PBS, and analyzed by flow-cytometry (Epics XL, Beckman Coulter, Fullerton, CA, USA). The mean PE fluorescence was calculated within the selected gate (G in Figure 5A) after 0 (no internalization, trypsin; Figure 5A, black line), 30 (Figure 5A, blue line) and 60 (Figure 5A, pink line) minutes of internalization. The percentage of endocytosis was calculated as the percentage of initial B3B4 binding (no internalization, no trypsin; Figure 5A, dashed line) after having subtracted the mean PE fluorescence at time 0 to each value (representing PE fluorescence not removed by trypsin). The data (means $\pm$ SE) presented are the values obtained from at least 3 independent experiments.

Characterization of stable MDCK cell lines

MDCK cell lines expressing *b*, *bd5* or mock transfected (control) were assessed for CD23 expression by incubating cells with the PE-B3B4 antibody as indicated above. Cells, were then washed in cold PBS-BSA, resuspended using a cell lifter (Costar) and analyzed by flow cytometry.

To characterize CD23 distribution at steady state in polarized cells, MDCK cell lines were grown on transwell filters for 6 days. The monolayers were then washed once in PBS and incubated with the mouse monoclonal IgE anti-DNP (Clone Spe-7 from Sigma; (1:100)) for 1 hour at 4°C in 1mM CaCl₂ and 1mM MgCl₂ containing PBS-BSA. Monolayers were washed, fixed and stained with a rabbit anti-ZO-1 antibody (Zymed Laboratories Inc, San Francisco, CA, USA), and a rat anti-mouse IgE antibody (1:50) (Southern Biotechnonolgy Associates, Birmingham, AL, USA) in permeabilization buffer. Cells were then incubated with an Alexa488-conjugated goat anti-rat IgG secondary antibody and a Cy3 conjugated donkey anti rabbit IgG antibody (Jackson ImmunoResearch Laboratories Inc, Soham, UK) as describe 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 the samples were analyzed by confocal microscopy (TCS SP2 AOBs, Leica).

Western Blotting

Proportional amount (1/100) of 2 days old media bathing both apical (7μl/ 500) and basolateral (21μl/ 1500) side of 6 days grown MDCK monolayers were analyzed by Western-Blotting using the NuPage system (Invitrogen). Briefly, samples were mixed with the LDS 4x sample buffer provided by the kit which lacks βmercapto-ethanol. The samples were not boiled before migration and proteins were separated on a 7% Tris-Acetate gel (Invitrogen) and transfer to PVDF membranes (Amersham Biosciences, Buckinghamshire, England) according to manufacturer's instructions (Invitrogen). Membranes were incubated with B3B4 antibody in Tris Buffer Saline (TBS)-Tween (0,05%), washed in TBS-Tween, and then incubated with an HRP-conjugated rabbit anti-rat IgG antibody (1:1000; Dakocytomation, Glostrup, Denmark).

To examine transcytosis of IgE, MDCK monolayers grown for 6 days on Transwell filters were washed twice in PBS and incubated for one hour at 37°C at the apical side with murine IgE (110μg/ml) in IgE binding media (DMEM 1 mM MgCl₂, 1mM CaCl₂) and at the basolateral side with DMEM supplemented with 1 mM MgCl₂. The effect of allergen on CD23-mediated transcytosis of IgE was tested by adding dinitrophenyl (DNP)-BSA (500 ng/ml; Molecular probes, Invitrogen) in the apical milieu together with the IgE for one hour at 37°C. Basolateral media from 4 Filters were then harvested, concentrated with the Microcon system (Millipore, Billerica, MA, USA), and subjected to western-blotting as describe above using the rat anti-mouse IgE antibody (1:500).

Detection of bound antibodies was performed with the ECL plus procedure (Amersham Biosciences).

Scatchard analysis

The affinity of IgE for CD23 splice forms was determined as previously described (12). Briefly, 293T cells were transiently transfected with CD23b or bD5 all contained in the pCR3.1 vector, using the FuGene 6 transfection reagent (Roche, Basel,

Switzerland). Transfections were performed in 6 well plates using the manufacturers recommended protocol for adherent cells. Forty eight hours after transfection, cells were collected and incubated with increasing concentrations of 125I-IgE (mouse anti-DNP IgE (38)), as described previously (12). After a 90 min incubation at 4°C, the cell bound radioactivity was determined by centrifugation through a phthalate oil cushion (12). Non-specific binding was determined by adding a 100-fold excess of unlabeled IgE to appropriate tubes and the nonspecific binding value was subtracted to give specific binding. Scatchard plots were used to examine whether the expected dual affinity binding was present.

Acknowledgments

This work was supported by grants from the Nutricia Research Foundation (A.B) and the Canadian Institutes of Health Research (M.H.P.). The authors would like to thank R. Madrid for helpful discussions and advices, people from the facilities of the Cochin Institute (confocal microscopy, DNA sequencing and flow cytometry) and J. Rappoport for critical reading of this manuscript.

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Figure legends

Figure 1: The *bd5* splice form binds IgE with high affinity.

293T cells were transiently transfected with CD23b (A) or *bd5* (B) encoding plasmids. Forty-eight hours after transfection, cells were collected and incubated with increasing concentrations of ¹²⁵I-IgE (mouse anti-DNP IgE) and cell bound radioactivity was determined as indicated in material and methods. The association constants (K_a) for both CD23b and *bd5* are indicated.

Figure 2: Schematic representation of CD23 splice forms.

The functional exonic organization of classical a and b and of the new CD23 splice form *bd5* is indicated. The primers couples used to amplify classical CD23 species or specifically the *bd5* splice have been represented to better explain the expected amplification products (see Fig. 3). (TM) Transmembrane, (Stalk) Stalk domain, (IgE) lectin like IgE binding domain.

Figure 3: The *bD5* splice form is induced by sensitization and IL-4

Total mRNA from jejunal fragments (A-C) from two control (lanes 1 and 2) and three sensitized mice (lanes 3-5) or from IEC-4 cells (D-F) untreated (lane 1) or treated for 24 or 48 hours with 20 or 10 ng/ml of IL-4 for (lanes 2 and 3 respectively) were isolated and reverse transcribed as described in Material and Methods. PCR were performed using primers couple designed to amplify CD23 (A and D), *bD5* splice form of CD23 (B and E) or ribosomal RNA 18S (C and F). Control PCR were done for each primers couple in which cDNA were omitted for the amplification reaction (-). One representative amplification reaction is shown out of at least three independent ones.

Figure 4: *bD5* undergoes constitutive internalization.

(A-D) HeLa cells transiently transfected with plasmids encoding *b* (A, B) or *bD5* (C, D) splice forms, were incubated with the B3B4 anti-CD23 monoclonal antibody for 1 h at 4°C. Transfected cells were then washed in cold PBS and incubated at 37°C for 30 minutes to allow internalization of the membrane-bound antibodies, in the presence of Alexa594-labelled transferrin to stain early endosomes. Cells were then washed, fixed, permeabilized and intracellular B3B4 antibody was revealed using an anti-rat Alexa488-labelled secondary antibody. (A, C) green fluorescence emitted by Alexa488 (CD23). (B, D) red fluorescence emitted by Alexa594 (transferrin). Insets show higher magnification of representative areas. Arrows stress colocalizing CD23 and transferrin dots. (E, F) HeLa cells were co-transfected with *bD5* and with GFP-2xFYVE encoding plasmids; the latter being targeted to PI3P-containing endosomal membranes. Transfected cells were fixed, permeabilized and stained for CD23 using the B3B4 monoclonal antibody followed by an Alexa594-conjugated secondary antibody. (E) red fluorescence emitted by Alexa594 (CD23). (F) Insets show higher magnification of representative areas. Arrows stress colocalizing CD23 and GFP-2xFYVE; arrowheads show CD23 positive structures only. Bars = 15 µm

Figure 5: Set up of a new quantitative endocytosis assay by flow cytometry.

(A) HeLa cells transiently transfected with *bD5* encoding plasmids were washed and cell surface associated CD23 was stained by a 1 hour incubation on ice with phyco-erythrin(PE)-labelled B3B4. Cells were then washed and incubated at 37°C for 0 (black line), 30 (blue line) or 60 (pink line) minutes. Cell surface associated CD23 was removed by treating the cells with trypsin for 4 minutes at 37°C (black, blue and pink). Cells were then resuspended and analyzed by flow cytometry. The maximum binding correspond to cells treated as for time 0 except the trypsin treatment (dashed line). (B) Values are expressed as a mean percent of the total cell-associated fluorescence (dashed line). Value of residual cell surface staining after trypsin treatment (black line) was removed for each time point. The data presented here for the *b* and *bD5* CD23 splice forms are representative of 3 independent experiments.

Figure 6: *bD5* is internalized via clathrin-coated pits.

(A-D) HeLa cells co-transfected with either $\mu 2$ or luciferase specific siRNA and with *bD5* encoding plasmid were analysed for CD23 endocytosis by fluorescence microscopy as described in Figure 4. Localization of internalized anti-CD23 antibodies (A and C) and controls of AP-2 expression (staining with the AP-6 anti α -adaptin monoclonal antibody (B and D)) are shown. Vesicular staining of internalized anti-CD23 is stress by arrows (A). Insets represent higher magnification of representative area of the plasma membrane showing dramatic decrease of punctate staining for AP-2 (B, D). Bar = 15 µm. (E) HeLa cells co-transfected with either $\mu 2$ or luciferase specific siRNA together with CD23b or *bD5* encoding plasmids were tested for B3B4 uptake by flow cytometry as described in Figure 5. Results are expressed as the mean percent of endocytosis compared to the control (luciferase, 100%). The data (means $\pm$ SE) presented here are the values obtained from at least 3 independent experiments.

Figure 7: *b* and *bD5* are localized at the apical membrane of polarized MDCK cell lines.

(A) MDCK cell lines either expressing *b* (blue) or *bD5* (pink) or non transfected (black), were incubated for 1 hour with B3B4-PE and then washed, resuspended in PBS and analyzed by flow cytometry. (B, C) MDCK cell lines expressing *b* or *bD5* grown for 6 days on Transwell filters were incubated from both apical and basolateral sides with mouse monoclonal IgE for one hour at 4°C, then washed, fixed and permeabilized. Bound IgE were revealed using a rat anti-mouse IgE followed by a goat anti-rat IgG conjugated to Alexa488 (green). Cells were also stained for ZO-1 a marker of tight-junctions using a polyclonal rabbit antibody followed by a Cy3-conjugated goat anti-rabbit antibody (red). x,y. (B) and y,z (C) sections are shown. Bar = 10 µm.

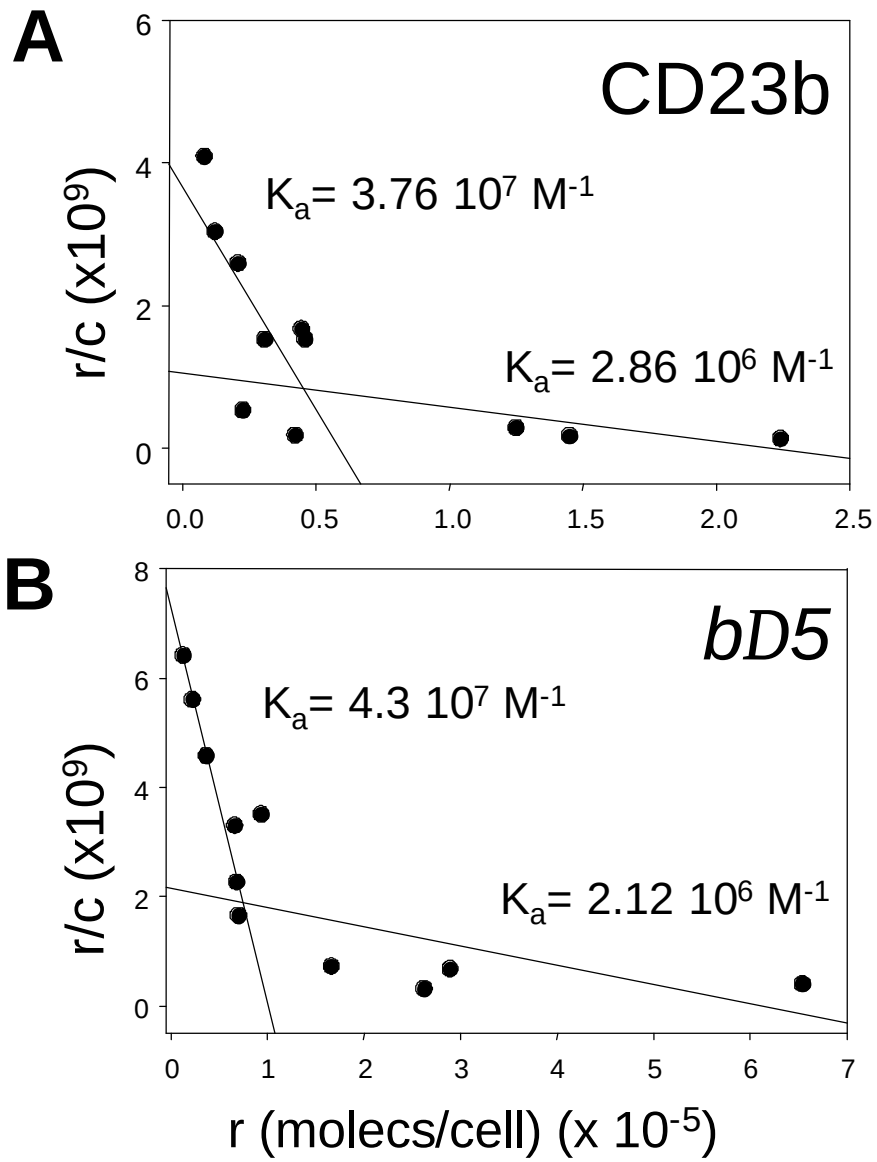
Figure 8: Soluble CD23 species are secreted at the apical side of polarized MDCK cells.

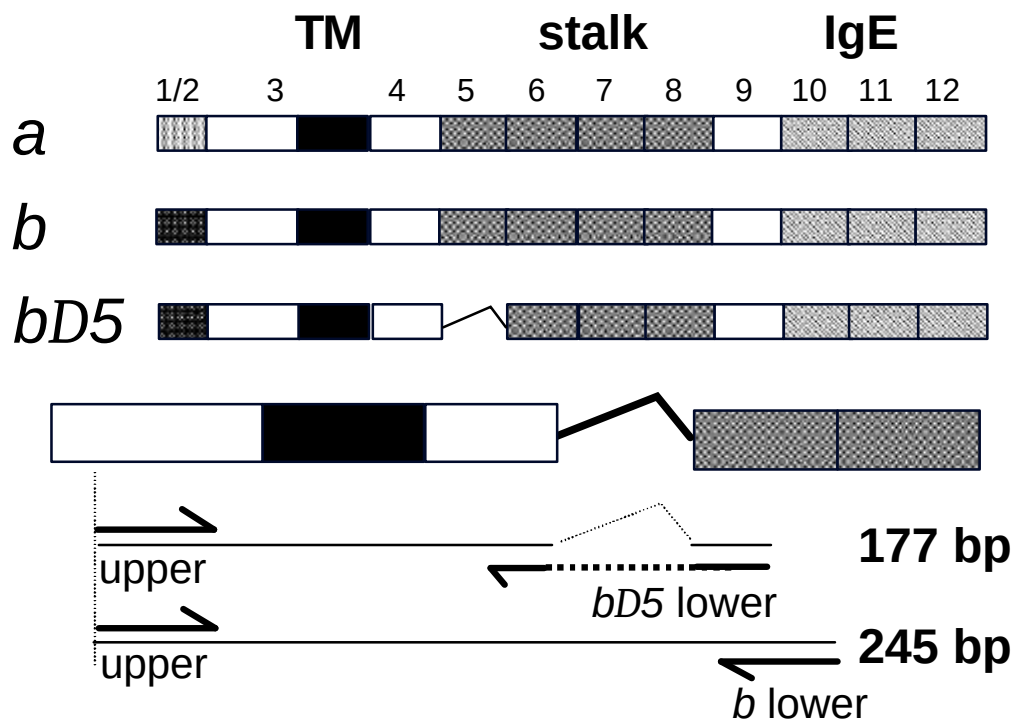
Media bathing apical (lanes 1, 3 and 5) and basolateral (lanes 2, 4 and 6) side of *b* (lanes 1 and 2) and *bD5* (lanes 3 and 4) expressing MDCK cell lines as well as of non transfected cells (lanes 5 and 6), were subjected to western-blotting with the B3B4 anti-CD23 antibody to detect soluble CD23 species. Monomers (*), dimers (°), trimers (#) and tetramers (•) of *b* (lane 1) and *bD5* (lane 3) are shown. Ap: apical medium; Ba: basolateral medium.

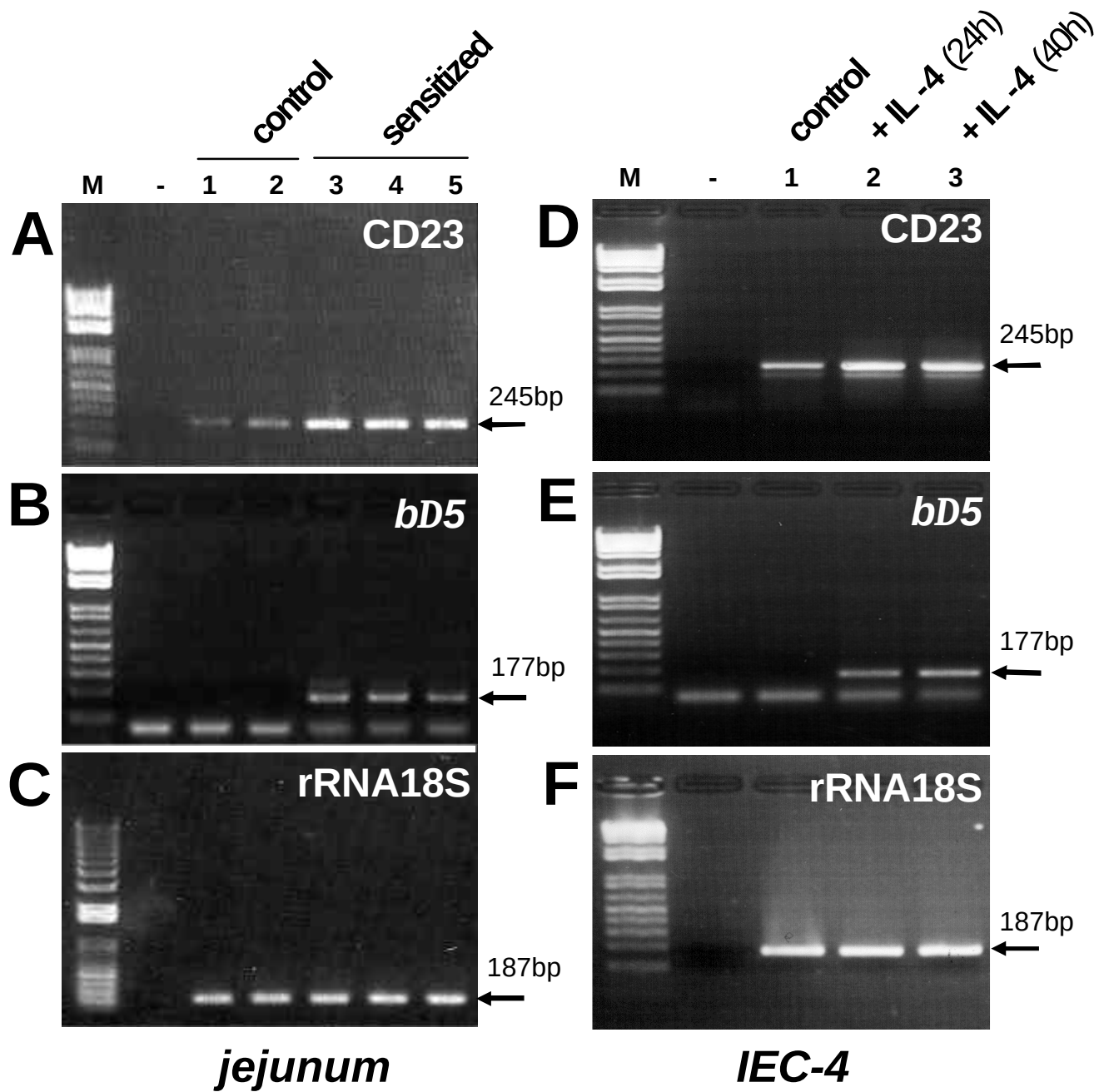
Figure 9: *b* and *bD5* are involved in different apical to basolateral transport activities.

Apically added free IgE (A) or IgE+ DNP-BSA complexes (B) were allowed to transcytose across non transfected MDCK or *b* or *bD5* expressing MDCK cell lines for 1 hour at 37°C. Basolateral media were then collected from 4 filters, concentrated and subjected to western-blotting using an antibody against mouse IgE. (C, D and E) After transport, filters used in B were fixed and process for immunofluorescence microscopy as in Figure 7 except that here IgE were detected using the rat anti-

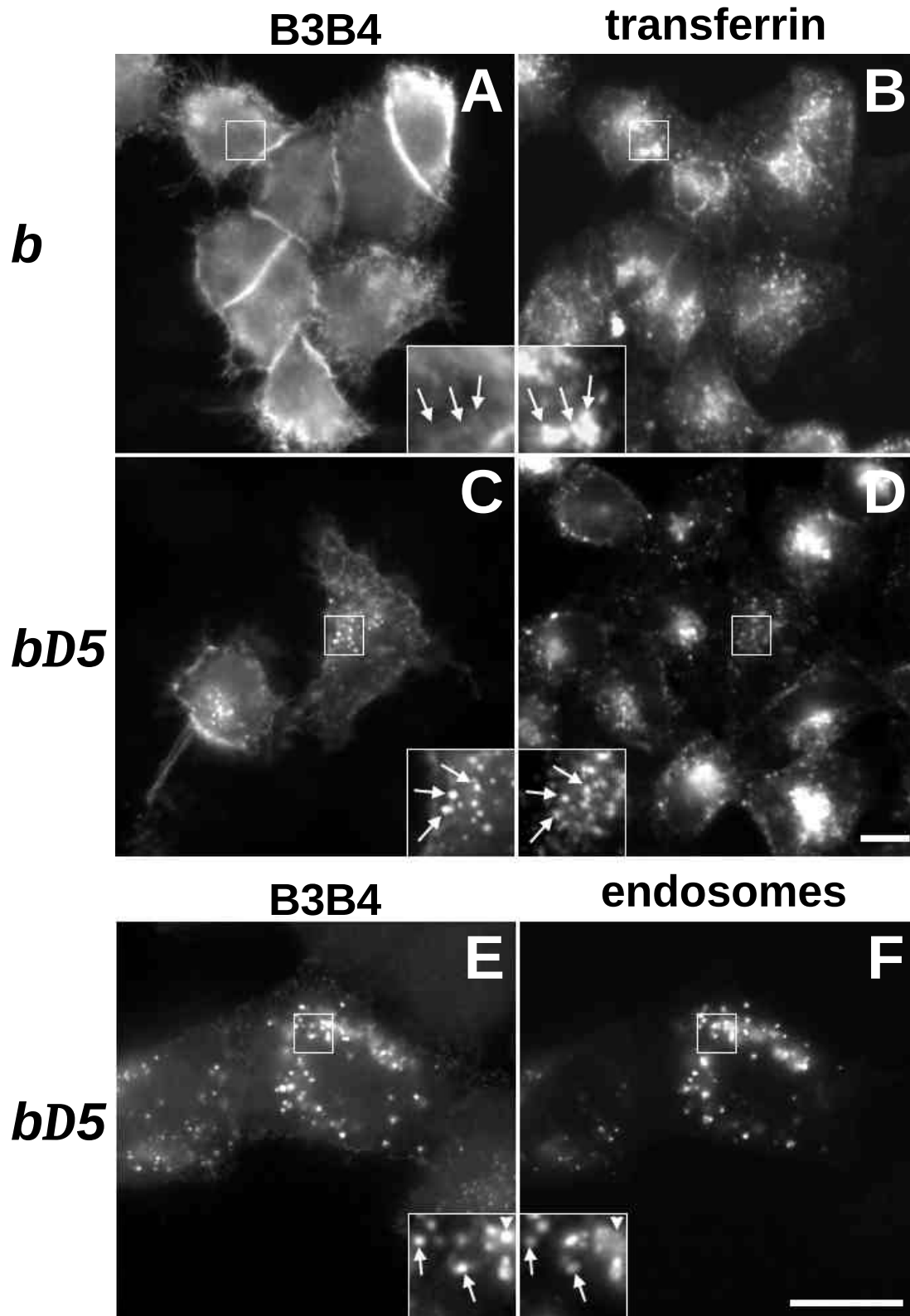
IgE antibody followed by an anti-rat secondary antibody. As in **B**, MDCK cell lines expressing *b* (**B**) or *bd5* (**C**) or non transfected MDCK cells (**D**) are compared. The pictures shown correspond to one of the four filters used in experiment described in **A**. The results shown are representative of at least 3 independent experiments. Bar = 15 μ m

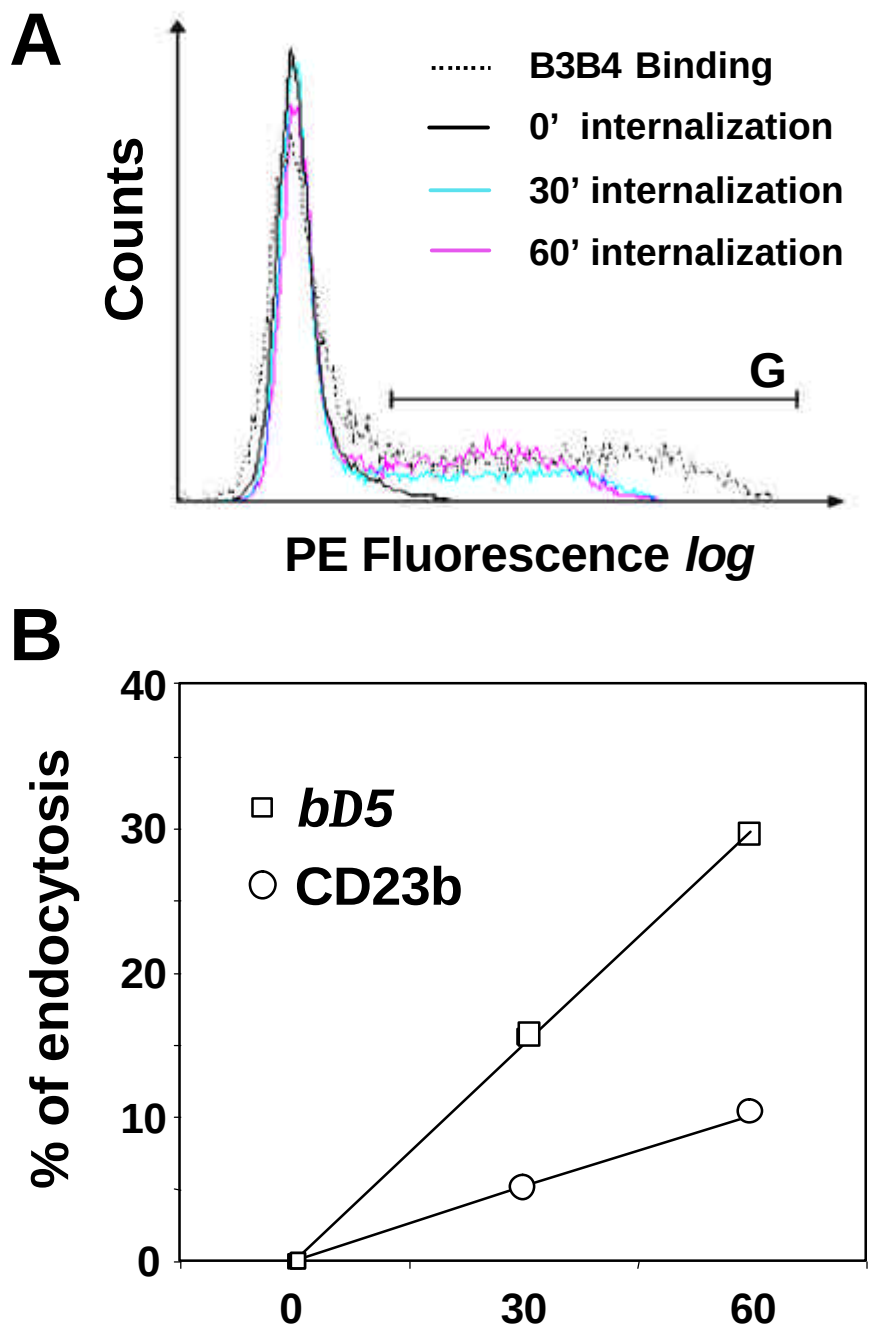




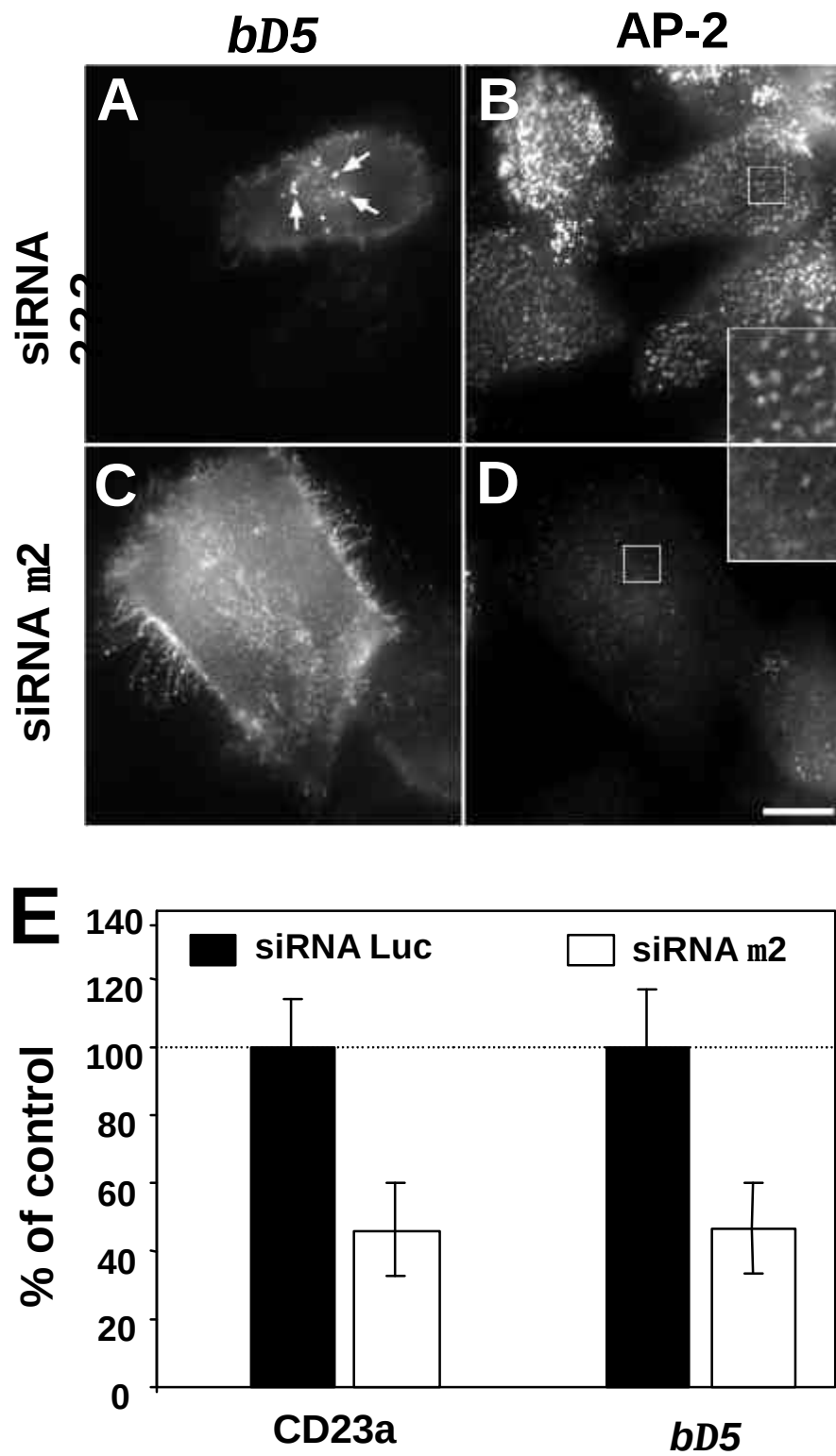


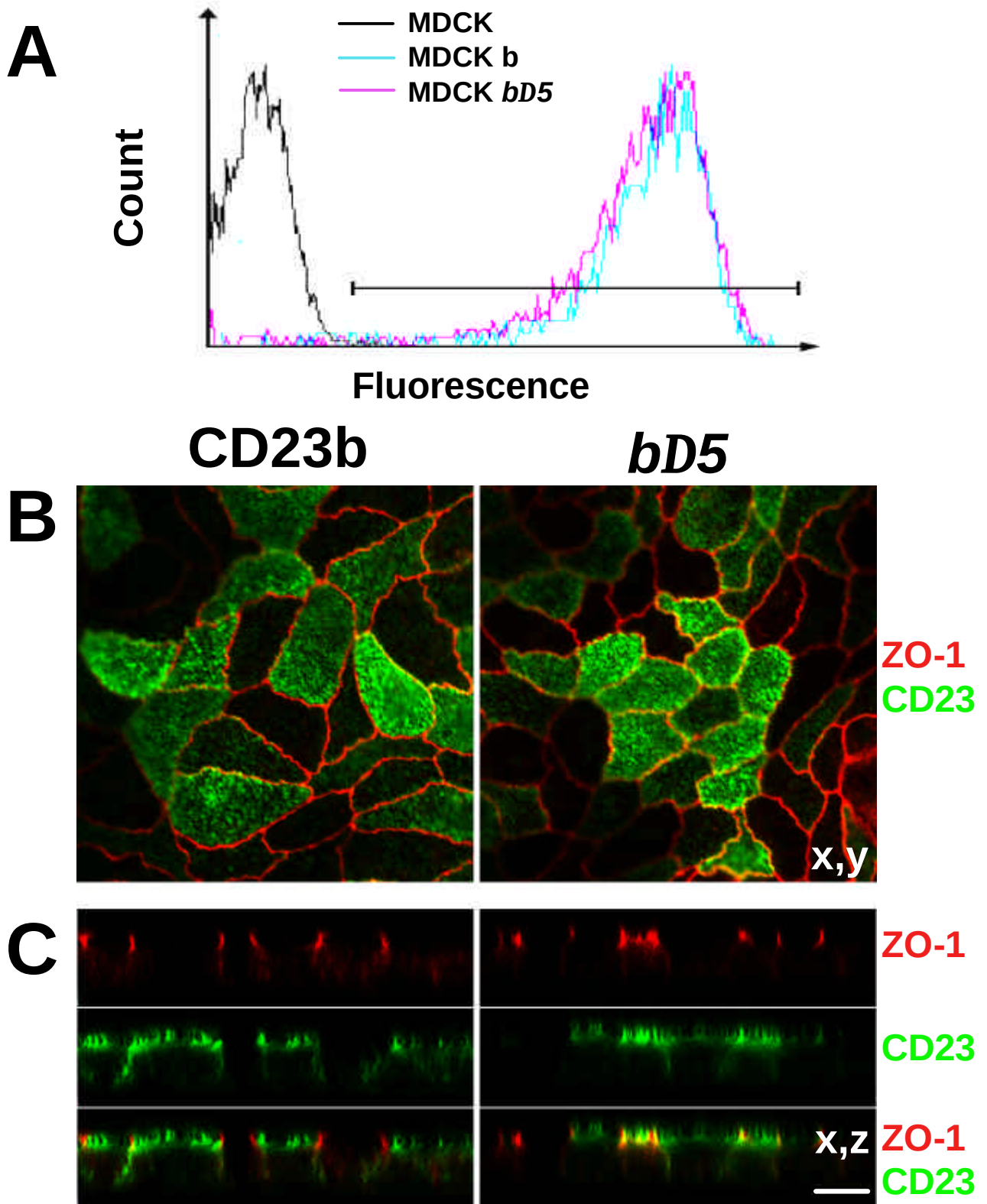
Montagnac G. et al, **Fig. 4**



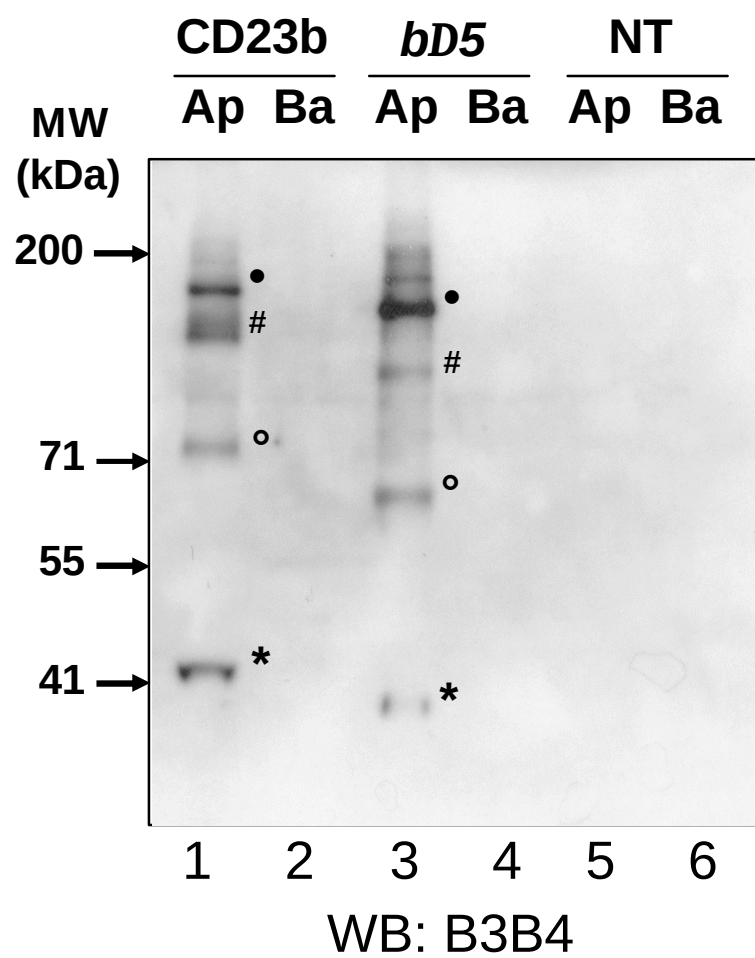


Montagnac G. et al, **Fig. 6**





Montagnac G. et al, **Fig. 8**



Montagnac G. et al, **Fig. 9**

