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Antibody-induced modulation of CD26 surface expression

T. MATTERN, C. REICH, M. DUCHROW, S. ANSORGE,* A. J. ULMER & H.-D. FLAD *Forschungsinstitut Borstel, Department of Immunology and Cell Biology, Borstel, and *Otto-v.-Guericke-Universität, Department of Experimental Immunology, Magdeburg, Germany*

SUMMARY

The ability of different anti-CD26 monoclonal antibodies to modulate the expression of CD26 on human T lymphocytes was investigated. By means of a new non-radioactive method using fluorescein isothiocyanate (FITC)-labelled and unlabelled anti-CD26 monoclonal antibodies and flow cytometry, we measured the internalization and re-expression of CD26 on freshly isolated resting human T lymphocytes. The modulation of CD26 surface expression takes place in primarily $CD26^+$ as well as in $CD26^-$ T lymphocytes, indicating the presence of an intracellular CD26 pool. In fact, with two different anti-CD26 monoclonal antibodies (Ta1 and M5) intracellular CD26 was detected out of which newly expressed CD26 might have originated. This intracellular CD26 pool appears to be maintained by continuous translation of CD26 mRNA.

INTRODUCTION

The T-cell activation marker CD26 is identical to the dipeptidyl peptidase IV (DPP IV),[1] a serine-type exopeptidase with a highly restricted substrate specificity, cleaving N-terminal dipeptides from polypeptides with proline or alanine as the penultimative amino acid.[2] The natural substrate of CD26 is still unknown, but there exist, with regard to their amino acid sequences, many substrates for this enzyme within the immune system, e.g. interleukin(IL)-1β, IL-2, IL-6, granulocyte colony-stimulating factor (G-CSF), lymphotoxin and substance P. Whether CD26 is involved in the degradation of these substrates, by for example enhancing or reducing their biological activity, is still unclear. In recent years it has become evident that CD26 is involved in the process of T-cell activation.[3–7] An adenosine deaminase (ADA) binding protein from the human kidney has been shown to be identical with CD26.[8] Furthermore, J. Kameoka (unpublished observations) have demonstrated a direct association of ADA with CD26 on human T lymphocytes. More recently, Callebaut *et al.*[9] proposed that CD26 may be a coreceptor of CD4 for human immunodeficiency virus (HIV) entry into the T cell.

Binding of antibodies to molecules on the cell surface can lead to a decrease of the particular surface molecule, a process termed antigenic modulation.[10] Antigenic modulation is often characterized by aggregation and internalization of antigen–antibody (Ag–Ab) complexes at 37°. It depends on the molecule and the epitope recognized by the antibody[11,12] as to whether or not modulation occurs. The consequences of modulation and internalization for the cell are hardly known. There may be some similarities between the internalization of Ag–Ab complexes and the internalization of peptides with their cell-surface receptors, as is known for transferrin and the transferrin receptor.[13,14] It has been shown for other surface molecules that binding of antibodies can initiate signal transduction events. Thus, T cells proliferate when CD2, CD3 or CD28 are cross-linked by antibodies.[15–17] The capacity for antibody-induced antigenic modulation may, therefore, be a property of those surface molecules involved in certain activation processes.

Since CD26 is known to be involved in T-cell activation, we have investigated whether anti-CD26 monoclonal antibodies (mAb) can modulate the expression of CD26. Employing a newly developed technique, we determined the internalization and re-expression of CD26 on resting T lymphocytes.

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Correspondence: Dr A. J. Ulmer, Forschunginstitut Borstel, Parkallee 22, D-23845 Borstel, Germany.

MATERIALS AND METHODS

Isolation of cells

Human peripheral blood mononuclear cells (MNC) were isolated from heparinized blood of healthy donors by density centrifugation on Ficoll–Paque, as described elsewhere.[18] T lymphocytes were isolated by adherence of MNC to nylon wool (Polysciences Inc., Eppelheim, Germany) according to the methods described by Julius *et al.*[19] The non-adherent cells consisted of more than 97% $CD2^+$ T lymphocytes.

Antibodies

The following anti-CD26 mAb were employed: M5,[20] M-A 261 (a kind gift of Dr P. E. Rieber, München, Germany), Ta1, Ta1–phycoerythrin (PE) and Ta1-fluorescein isothiocyanate (FITC) (Coulter, Krefeld, Germany). Unlabelled mAb were detected with FITC- or PE-labelled $F(ab')_2$ fragments of goat anti-mouse IgG (GAM–FITC; Jackson Immuno Research Laboratories, West Grove, PA).

Flow cytrometric analysis

Flow cytrometric analysis was performed by using a Cytofluorograf (System 50H; Ortho Diagnostic System Inc., Westwood, MA). Cells with a mean fluorescence intensity between channel 0 and 10 were defined as $CD26^-$ T lymphocytes. Cells with a mean fluorescence intensity between channel 100 and 1000 were regarded as $CD26^+$ cells. Less than 5% of the cells had fluorescence intensities between channel 10 and 100, so that it was easy to disguingish between $CD26^-$ and $CD26^+$ cells. A comparison of mean fluorescence intensities of all cells was not possible in our test system, because the use of direct-labelled mAb and unlabelled mAb resulted in different mean fluorescence intensities but not in different absolute numbers of $CD26^+$ or $CD26^-$ cells.

Modulation studies

Measurement of shedding. Cells were stained with a FITC-conjugated anti-CD26 mAb (Tal–FITC) in phosphate-buffered saline (PBS) for 20 min at 4°. Unbound mAb was removed by washing the cells with PBS. Cells were then resuspended in prewarmed (37°) medium [RPMI-1640, 10% fetal calf serum (FCS)] and incubated for different intervals at 37°, as indicated in the legends. Following this incubation, cells were washed and fixed by adding paraformaldehyde. The reduction of CD26 expression after the incubation period should be the result of the shedding of CD26 (Fig. 1), while in the case of internalization (cell membranes are transparent for intracellular staining) the number of $CD26^+$ cells should remain stable over a long incubation period until the labelling decreases due to enzymatic degradation of the fluorescence dye. With these staining properties there is no distinction between 'no modulation' and 'internalization'.

Measurement of internalization. Cells were stained with an unlabelled anti-CD26 mAb in PBS for 20 min at 4°. Unbound mAb was removed by washing the cells with PBS. Cells were then resuspended in prewarmed medium (RPMI-1640, 10% FCS) and incubated for different intervals at 37°. After this incubation, cells were washed and fixed by adding paraformaldehyde. Remaining mAb on the cell surface were detected by staining cells with GAM–FITC. A reduction of $CD26^+$ cells should be the result of shedding or internalization (Fig. 1).

Measurement of newly expressed antigen. Cells were stained with an unlabelled anti-CD26 mAb in PBS for 20 min at 4°. Unbound mAb was removed by washing the cells with PBS. Cells were then resuspended in prewarmed medium and incubated for different intervals at 37°. After this incubation, cells were fixed by adding paraformaldehyde. Newly expressed CD26 was detected by staining cells with a direct-labelled anti-CD26 mAb (Tal–FITC) (Fig. 1).

Measurement of modulation of CD26 on $CD26^+$ and $CD26^-$ T cells. After isolation of $CD26^+$ and $CD26^-$ T cells by cell sorting using a Cytofluorograf, the cells were stained with Tal–FITC for 20 min at 4°. Unbound antibodies were removed by centrifugation over a FCS gradient. Cells were then resuspended in prewarmed medium and incubated for different intervals at 37°. After this incubation, the cells were fixed by adding paraformaldehyde. Newly expressed CD26 on $CD26^-$ sorted T lymphocytes was measured by staining with Tal–PE. Expression of CD26–Tal–FITC complexes remaining on the surface of $CD26^+$ sorted cells was determined with GaM–PE. Newly expressed CD26 was detected by staining with Tal–PE.

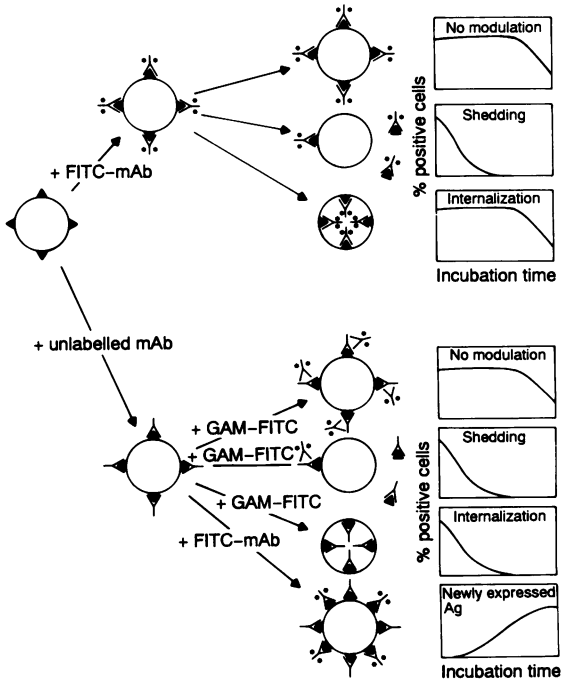

Figure 1. Theoretical model for the measurement of CD26 surface modulation with different staining strategies. For measurement of shed Ag–Ab complexes, cells were stained with a direct-labelled anti-CD26 mAb (upper part) washed, and then incubated at 37° for different times. When the Ag–Ab complexes were shed, the number of cells stained with the FITC–labelled mAb should decrease during the incubation at 37°. With this method it is not possible to distinguish between no modulation and internalization, since in both cases the number of $CD26^+$ cells should be constant over a long incubation period (intracellular anti-CD26–FITC is also detectable by flow cytometry). The number of positive cells might then decrease due to enzymatic degradation of the fluorescence dye. In a second staining protocol, cells were stained with an unlabelled anti-CD26 mAb (lower part). After incubation at 37°, antibodies remaining on the cell surface were detected with GAM–FITC. In the case of no modulation, the number of cells should again be constant over a long incubation period. With this method it is not possible to distinguish between shedding and internalization, since in both cases the number of $CD26^+$ cells should decrease during the incubation. But if shedding of the Ag–Ab complexes could be excluded with the upper staining procedure, a decrease of $CD26^+$ cells should be the result of the internalization of these complexes. For newly expressed CD26, cells were stained with an unlabelled anti-CD26 mAb, washed, and then incubated at 37°. Newly expressed CD26, not occupied be the unlabelled mAb, could now be detected by a FITC-labelled anti-CD26 mAb.

Measurement of intracellular CD26. Cells were incubated with 0·4 mg/ml digitonin (Sigma, Deisenhofen, Germany) in PBS at room temperature for 30 min. After washing the cells twice with PBS, they were stained for 20 min with different CD26 mAb. Unbound mAb was removed by washing three times with PBS. Unlabelled CD26 mAb was determined by staining with GAM–FITC. Unbound GAM–FITC was also removed by washing three times in PBS. After fixation, cells were analysed in a Cytofluorograf. By use of this staining protocol intracellular and extracellular CD26 could be detected.

Detection of CD26 mRNA by polymerase chain reaction (PCR)

Freshly isolated, unstimulated $CD26^-$ and $CD26^+$ T cells

(1×10^6/ml) were washed, spun down, and the pellet frozen at −70° until further analysis. The preparation of mRNA was performed according to Thanhäuser *et al.*[21] by means of oligo-dT-coated magnetic beads (Dynal AS, Oslo, Norway). Synthesis of cDNA was performed with oligo-dT_{17} as primer for reverse transcriptase (Superscript; Gibco-BRL, Eggenstein, Germany). PCR was carried out in an automatic DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, USA). The step-cycle program was set to denature at 94° for 1 min, to anneal at 55° for 1 min, and to extend at 72° for 1·5 min for a total of 35 cycles. For each sample a β-actin PCR served as control for successful mRNA preparation. PCR products were run on agarose gel containing 0·01 μg/ml ethidium bromide.

RESULTS

Anti-CD26 antibodies induced the modulation of CD26 in freshly isolated T cells

The newly developed staining technique allowed the distinction to be made between shedding or internalization of Ag–Ab complexes and re-expression of an antigen on the cell surface. When cells were stained with a labelled antibody (Ta1–FITC) and incubated at 37° in medium to allow metabolic reactions, a decrease of the number of stained cells indicated shedding of Ag–Ab complexes (Fig. 1). Since the number of $CD26^+$ cells remained stable during the incubation in medium at 37° (a typical result of such an experiment is shown in Fig. 2), shedding of Ag–Ab complexes could be excluded.

A further staining procedure was employed to investigate whether or not Ag–Ab complexes were internalized. For this purpose, cells were stained with unlabelled CD26 mAb and incubated at 37°. When the cells were subsequently stained with GAM–FITC antibodies, only residual anti-CD26 mAb on the cell surface could be detected, since the GaM-antibodies do not penetrate the cell membrane. Figure 2 shows a typical experiment where the anti-CD26–CD26 complexes were internalized, since the number of cells stained with GAM–FITC was diminished. A loss of CD26–anti-CD26 complexes by shedding could be excluded by the finding that CD26 was still detectable by FITC-labelled anti-CD26 mAb. This internalization of surface CD26–anti-CD26 complexes occurred rapidly, and after 30 min of incubation only half of the initial number of $CD26^+$ cells could be detected. After 2–4 hr the cell surface was almost devoid of Ag–Ab complexes.

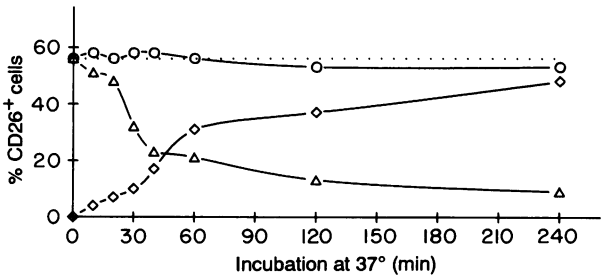

Figure 2. Modulation of CD26 surface expression on freshly isolated resting T lymphocytes. Freshly isolated human T cells were stained with the direct-labelled anti-CD26 mAb Ta1–FITC, or with unlabelled Ta1, at 4°. After removing of unbound antibodies by washing with PBS, cells were incubated in culture medium (RPMI-1640, 10% FCS) at 37° for the indicated intervals. For shedding, Ta1–FITC-labelled cells were measured after this incubation in a Cytofluorograf. For internalization, cells were first stained with an unlabelled Ta1, incubated at 37° and then labelled with GAM–FITC. For newly expressed CD26, Ta1-labelled cells were stained after the incubation at 37° with Ta1–FITC and then measured. For a control experiment for this modulation, cells were kept at 4° during the time of modulation. Data are expressed as number of stained cells (%). (○) Shedding (direct-labelled mAb); (△) internalization (unlabelled mAb plus GAM–FITC); (◇) newly expressed CD26 (unlabelled mAb + direct-labelled mAb); (- - -) control.

With a third staining strategy, newly expressed CD26 could be detected on the surface of T cells. Therefore, cells were labelled again with an unconjugated anti-CD26 mAb (Ta1). After incubation at 37°, cells stained with labelled Ta1–FITC instead of GAM–FITC (Fig. 1). This antibody bound to newly expressed CD26 because primary CD26 was occupied by unlabelled Ta1. This re-expression occurred very quickly and after 30–120 min half of the initial number of cells expressed new CD26 on their surface (Fig. 2). In three independent experiments, the number of cells with newly expressed CD26 on their surface reached, but never exceeded, the level of surface expression prior to internalization within 1–3 hr of incubation. The same modulation of CD26 surface expression (internalization and re-expression) could be observed with the anti-CD26 mAb M5 and M-A 261 (data not shown). Control experiments for this modulation were performed with PBS supplemented with 1·5% sodium azide instead of using the incubation medium, or with cells kept at 4° during the time of modulation. Internalization of CD26–anti-CD26 complexes and re-expression of CD26 could not be observed under these conditions (data not shown).

Modulation and re-expression of CD26 on $CD26^-$ and $CD26^+$ T cells

Next we investigated the modulation of CD26 on $CD26^-$ and $CD26^+$ T cells, isolated by cell sorting using FITC-labelled Ta1. After incubation for different times in medium at 37°, $CD26^-$ T cells were stained with the Ta1–PE. Figure 3a shows the typical results of such an experiment. $CD26^-$ T cells (initially containing less then 2% $CD26^+$ T cells) started to express CD26 on their surface. Within 1 hr of incubation at 37°, $CD26^+$ T cells were found, and after 8 hr nearly the same amount of $CD26^+$ T cells was detectable as in the $CD26^+$ T-cell subset. The number of positive T cells did not increase any further, even after incubation for 2 days (data not shown). The modulation of CD26 surface expression on initially $CD26^+$ T lymphocytes was determined by two staining strategies. Residual CD26–Ta1–FITC complexes on the surface of the cells were detected by staining with GAM–PE. As shown in Fig. 3b, all CD26–Ta1–FITC complexes were internalized within 24 hr. Newly expressed CD26 on $CD26^+$ sorted T lymphocytes was located by staining with labelled Ta1–PE. The kinetics of CD26 reexpression on $CD26^+$ sorted T lymphocytes were similar to those of $CD26^-$ T lymphocytes.

Intracellular CD26

Because of the fast re-expression of CD26 on the surface, we supposed that T cells may have an intracellular CD26 pool. To

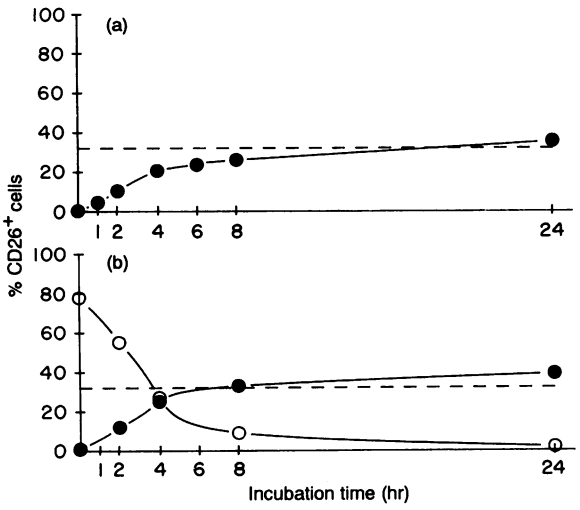

Figure 3. Modulation of CD26 surface expression on $CD26^+$ and $CD26^-$ T lymphocytes. Freshly isolated T lymphocytes were labelled with Ta1–FITC and sorted for $CD26^-$ (a) and $CD26^+$ (b) cells. Sorted cells were incubated in culture medium at 37° for the indicated time intervals. Remaining complexes of CD26–Ta1–FITC on the surface of $CD26^+$ sorted T lymphocytes were detected by staining with GAM–FITC (b; ○). Newly expressed CD26 on $CD26^+$ as well as on $CD26^-$ T lymphocytes was detected with direct-labelled Ta1–PE (a + b; ●). (- - -) CD26 before sorting.

verify this assumption, T cells were incubated with digitonin to make the cell membrane permeable for antibodies. As shown in Table 1, in a representative experiment, intracellular CD26 was detectable with the mAb M5 and Ta1, but not with the mAb M-A 261, in both unsorted and $CD26^-$ T cells. About 30% of the $CD26^-$ cells could be stained with Ta1 or M5.

Expression of CD26 mRNA on resting $CD26^-$ and $CD26^+$ T cells

$CD26^+$ and $CD26^-$ T cells isolated by cell sorting were examined for the presence of CD26 mRNA. As shown in Fig. 4, CD26 mRNA was detected in unstimulated T cells. Furthermore, CD26 mRNA was found in both $CD26^+$ and $CD26^-$ T cells.

DISCUSSION

Internalization and re-expression of cell-surface CD26 on human T lymphocytes was demonstrated using FITC-labelled and unlabelled anti-CD26 mAb (Fig. 2). Rates of internalization and re-expression were nearly equivalent. These processes of internalization and re-expression of CD26 on the cell surface depended on metabolically active cells, because neither internalization nor re-expression could be observed at 4° (Fig. 2) or in the presence of sodium azide (data not shown). Internalization and re-expression of CD26 on the cell surface were found in both $CD26^+$ T lymphocytes (Fig. 3b) and $CD26^-$ T lymphocytes. A part, but not all, of the $CD26^-$ cells altered to $CD26^+$ during the incubation at 37° (Fig. 3a). Whether CD26 expression in such $CD26^-$ cells is an effect also found *in vivo* is still under investigation. However, it has been shown that incubation of T cells over 96 hr did not alter CD26 expression significantly.[22] Interestingly, $CD26^+$ T lymphocytes reached, but never exceeded, the individual number of $CD26^+$ cells for each donor observed before sorting. However, it should be mentioned that each individual donor had a different number of $CD26^+$ cells.

Newly expressed CD26 might be derived from an intracellular CD26 pool. Such intracellular CD26 could be detected in unsorted as well as in sorted $CD26^-$ T lymphocytes with the antibodies Ta1 and M5, but not with the mAb M-A 261 (Table 1). A possible explanation for this finding might be that the mAb M-A 261 recognizes a glycosylated form of CD26 only present on the cell surface, not intracellularly. This intracellular CD26 pool seems to be maintained by permanent translation of CD26 mRNA, since CD26 mRNA was detected in freshly isolated, unstimulated $CD26^-$ as well as $CD26^+$ T lymphocytes (Fig. 4).

Whether an internalized CD26 molecule is degrated immediately by endosomal or cytosomal enzymes, or whether it could recycle to the cell surface, could not be investigated

Table 1. Intra- and extracellular expression of CD26

	Unsorted T cells		$CD26^-$ T cells	
Antibodies for labelling	% extracellular CD26	% intra- and extracellular CD26	% extracellular CD26	% intra- and extracellular CD26
M5 + GAM–FITC	25·0 (193)	92·0 (388)	1·1 (241)	32·0 (268)
Ta1 + GAM–FITC	37·0 (206)	86·0 (339)	0·9 (212)	26·0 (313)
M-A 261 + GAM–FITC	40·0 (222)	34·0 (225)	1·3 (197)	2·3 (231)
Unlabelled control	0·2 (201)	0·3 (187)	0·2 (198)	0·3 (199)
GAM–FITC	1·0 (187)	7·0 (160)	0·2 (187)	0·3 (199)

Freshly isolated T cells or $CD26^-$ T cells (isolated by sorting with Ta1–FITC) were stained before (= extracellular CD26) or after digitonin treatment (0·4 mg/ml digitonin, 30 min, 20°; = intra- and extracellular CD26) with the indicated antibodies. After removal of unbound antibodies, cells were measured in a Cytofluorograf. Data are expressed as the percentage number of stained cells. Mean fluorescence intensity of positive cells is given in parentheses.

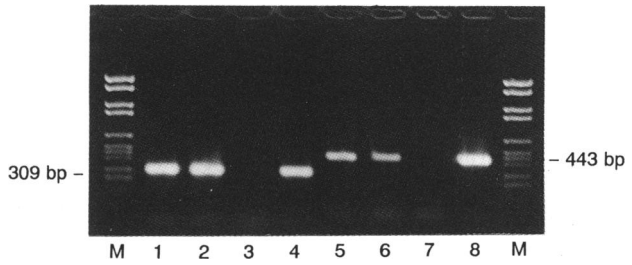

Figure 4. Reverse transcriptase (RT)—PCR of $CD26^+$ and $CD26^-$ sorted T cells directly after isolation. For details see the Materials and Methods. M, molecular weight marker VI (pBR328 DNA × *Bgl*1 + pBR328 DNA × *Hinf*1; Boehringer, Mannheim, Germany). Lanes 1–4: β-actin PCR; lane 1, $CD26^+$ T lymphocytes; lane 2, $CD26^-$ T lymphocytes; lane 3, water control; lane 4, positive control [phytohaemagglutinin (PHA) blasts]; lanes 5–8, CD26 PCR; lane 5, $CD26^+$ T lymphocytes; lane 6, $CD26^-$ T lymphocytes; lane 7, water control; lane 8, positive control (PHA blasts). Poly (A) + RNA was isolated from 1×10^6 cells by means of oligo-dT-coated magnetic beads. The cDNA synthesis was performed with an oliga-dT_{17} primer. Two microlitres of cDNA was used as template in a standard 50 μl PCR with the primers $CD26^+$, CTCTGCTCTGTGGTGGTCTC; $CD26^-$, GCCTAAATCTTCCAACCCAG; β-$actin^+$, AGCGGGAAATCGTGCGTG; β-$actin^-$, CAGGGTACATGGTGGTGGTGCC. The corresponding PCR products were 443 bp for CD26, and 309 bp for β-actin.

with this method. However, for human hepatocytes[23–27] and in intestinal epithelial cells[28] it has been shown that CD26 is recycled. These authors could demonstrate different half-lives of the carbohydrate and protein moieties of CD26. It is thought that recycling of CD26 is presumably a constitutive process that reflects repair mechanisms for the oligosaccharide chains of CD26.[27] The addition of an antibody to a cell-surface component might influence the kinetics of internalization or synchronize the internalization.[29]

Recycling of CD26 may also indicate a receptor function of this glycoprotein apart from its enzymatic activity. Recently, it has be shown that an ADA binding protein in human kidney is identical with CD26.[8] Binding of ADA to CD26 on human T lymphocytes could be demonstrated.[30] Furthermore, ADA infused into rabbits was shown to bind to a receptor protein and to be internalized into cells of the proximal renal tubule together with its receptor, indicating CD26 to be the ADA receptor.[31] Time kinetics of this internalization of ADA are in accordance with our own observations. Forty-five minutes after infusion of ADA through the ear vein of rabbits, this enzyme could be detected in the cytoplasm of the cells of the proximal renal tubules.[31] Binding of anti-CD26 mAb to CD26 might mimic the binding of ADA to its receptor and thereby induce internalization of the Ag–Ab complexes. Taken together, these results indicate that CD26 is modulated (internalized and re-expressed) on the surface of human T lymphocytes. Whether this modulation is induced by cross-linking with anti-CD26 mAb, or is an effect of mAb binding alone, or even may occur spontaneously, is still under investigation. Newly expressed CD26 may be derived as a product of CD26 mRNA, which is constitutively present in all T cells, as well as from previously internalized CD26. The biological significance of this remodelling of CD26 remains to be investigated.

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