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*J Immunol* 2002; 168:5612-5620; doi: 10.4049/jimmunol.168.11.5612
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AP2 Adaptor Complex-Dependent Internalization of CD5: Differential Regulation in T and B Cells

Xianghuai Lu, Robert C. Axtell, James F. Collawn, Andrew Gibson, Louis B. Justement, and Chander Raman

CD5 is a key regulator of Ag receptor-mediated activation, selection, and differentiation in both T and B cells. Accumulating evidence indicates that lymphocyte activation and selection are sensitive to variations in the cell surface expression levels of CD5. We now show that CD5 expression on the surface of B cells is regulated posttranscriptionally by direct interaction with the μ chain subunit of the AP2 adaptor complex, which links transmembrane proteins to clathrin-coated pits. CD5 is rapidly internalized from the cell surface in lymphoid cell lines and mature splenic T and B cells, and peritoneal CD5+ B cells following monovalent or bivalent ligation of the receptor. We mapped the μ2 subunit binding site on CD5 to Y429 and determined that the integrity of this site was necessary for CD5 internalization. Cross-linking of the Ag receptor with intact Abs inhibited CD5 internalization in B cells, but had the opposite effect in T cells. However, if F(ab')2 Abs were used to stimulate the Ag receptor in B cells, the effect on CD5 internalization was now similar to that observed in T cells, indicating that signals through the Ag receptor and FcR regulate CD5 endocytosis in B cells. This was confirmed using an FcyRIIB1-deficient B cell line. The ability to differentially alter posttranslational CD5 expression in T and B cells is likely to be key in regulation of Ag receptor signaling and generation of tolerance in T and B lymphocytes. The Journal of Immunology, 2002, 168: 5612–5620.

CD5 is a 67-kDa glycoprotein belonging to the scavenger receptor family of proteins that is constitutively expressed on all T cells and a subset of B cells known as B1a B cells (1–4). Structurally, CD5 is closely related to CD6, each having three extracellular scavenger receptor cysteine-rich (4) domains (5). Although the identity of a CD5 counter receptor/ligand remains controversial, potential ligands include CD72 (6), gp40–80 (7, 8), and Ig-framework region sequences (9). A good candidate for a CD5 ligand is a 150-kDa glycoprotein that is expressed on monocytes, lymphocytes, and from proteolytic cleavage in serum (10).

CD5 is associated both physically and functionally with the Ag receptor family of proteins that is constitutively expressed in both T and B cells. Based on data from CD5-deficient mice, it has been suggested that CD5 exerts a net negative effect on TCR signaling in T cells (11, 12). Immature T cells in CD5−/− mice are hyperresponsive to TCR stimulation and exhibit altered positive and negative selection (11). However, other studies suggest that CD5 stimulation enhances negative selection in CD4+ or CD8+ single positive semimature thymocytes (13, 14). In mature T cells, CD5 is primarily considered to be a costimulatory molecule. Costimulation experiments indicate that CD5 signaling is synergistic with TCR/CD3 or CD28 stimulation in T cells from mice or humans (15–17).

In contrast to T cells, very little is known regarding the function of CD5 in B cells. As with T cells, the data indicate that the effect of CD5 on B cell Ag receptor (BCR)4-mediated signaling can be inhibitory or costimulatory. The study showing that B1a (CD5+) B cells can be induced to proliferate in response to anti-μ stimulation when CD5 is sequestered away from the BCR or deleted as in B1 B cells from CD5-deficient mice indicates an inhibitory role for CD5 (18). This conclusion is also supported by the observation that coligation of CD5 and BCR leads to inhibition of calcium mobilization, and this inhibitory effect requires the pseudo-immureceptor tyrosine-based activation motif in the cytoplasmic tail of CD5 (19). However, B cell expansion and splenic hyperplasia associated with elevated CD5 expression as in aged CD5+ or CD19 transgenic mice is consistent with a stimulatory role for CD5 in the B lineage (20, 21). Overall, these data suggest that the net effect of CD5 signaling is likely to be contextual and dependent on the differentiation and/or activation stage of the cell.

In both the B and T cell compartments, the expression of CD5 in vivo is directly associated with strength of Ag receptor-induced intracellular signals (22–24). CD5 expression and the frequency of CD5+ B cells is elevated in mice carrying naturally occurring or induced mutations that enhance the strength of BCR-induced intracellular signals (21, 25–27). In contrast, mutations that lead to diminished ability to signal via the BCR result in either a dramatic decrease in the number of CD5+ B cells or a complete loss of this population (28, 29). Similarly, the level of CD5 expressed on T cells is altered in mice that have mutations that affect the strength of TCR/CD3-mediated signals (30). These observations suggest that the regulation of CD5 expression is likely to be a key event in

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Received for publication February 7, 2001. Accepted for publication April 8, 2002.

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2 This work was supported by grants from Arthritis Foundation AI, National Institutes of Health Grants AI6221 and AI44836 (to C.R.), the Cystic Fibrosis Foundation (COLLAWGO), and the American Heart Association (Established Investigator Grant; to J.F.C.).

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0022-1767/02/$02.00
the maintenance of homeostasis in these lymphoid compartments. Although CD5 expression is regulated at the transcriptional level (31), it appears that posttranslational processes also regulate the expression of CD5 (20). The posttranslational regulation of CD5 expression is likely to be involved in the regulation of early signaling initiated by Ag receptor ligation in T and B cells. We show that CD5 is rapidly removed from the cell surface at a rate significantly greater than constitutive turnover of the receptor. To identify the proteins that may be involved in this process, we used the yeast two-hybrid system and screened a human PBL library with the cytoplasmic tail of CD5 as “bait”. Using this approach, we determined that the μκ (AP50) subunit of the AP2 adaptor complex associates with CD5. This finding was confirmed based on coimmunoprecipitation experiments using cell lines. Subsequently, the AP2 binding site was mapped to a tyrosine-based internalization motif, which contains the first tyrosine of the pseudo-immunoreceptor tyrosine-based activation motif, and we determined that this motif was required for CD5 internalization. We show that CD5 internalization was enhanced by Ag receptor signaling in primary T cells, but inhibited in B cells. The inhibition of CD5 internalization in B cells was dependent on cofilination of F-cort.

Materials and Methods

Mice

C57BL/6/CD5 transgenic mice (20) were a gift from J. F. Kearney (Department of Microbiology, University of Alabama, Birmingham, AL) and BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used at 9–16 wk of age. The CD5 gene in C57B/c transgenic mice is under the control of the IgH chain promoter and enhancer.

Cell lines and reagents

The human T leukemia cell line, Jurkat, murine T cell line, EL4, and B cell lines, A20 and IA1.6, were maintained in RPMI 1640 medium supplemented with 10% FCS (Life Technologies, Gaithersburg, MD). COS-7 cells were grown in DMEM supplemented with 10% FBS. Monoclonal anti-ag5-adaptin (AP-2a, clone 100/2) and monoclonal IgG1 (MOPC21) were obtained from Sigma-Aldrich (St. Louis, MO). PE anti-human CD5 (UCHT2), anti-human CD3 (UCHT1), PE anti-mouse CD90.2 (Thy 1.2, G7), FITC anti-mouse CD19 (1D3), biotin anti-mouse IgG1 (IgG-4a), and streptavidin-allophycocyanin were obtained from BD Pharmingen (San Diego, CA). Anti-CD3 (145-2C11) and anti-CD5 (53-7.313) mAb were from Bio-Rad, and SuperSignal chemiluminescence substrate was obtained from Pierce. Peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Life Technologies, and SuperSignal chemiluminescence substrate was obtained from Pierce. Peroxidase-conjugated goat anti-mouse IgG was from Jackson Immunoresearch Laboratories (West Grove, PA).

Yeast two-hybrid screen

The yeast two-hybrid screen was performed using the GAL4 binding domain (BD) CD5 cytoplasmic domain (BD-CD5) fusion construct as described previously (32).

Constructs

To generate BD-CD5 deletion mutants, we used the QuikChange mutagenesis kit from Stratagene (La Jolla, CA) as described previously (32). The eight primers used to generate all the mutations are as follows: 1) 5'-CCCGACGAGCTGTACGTCA0TG-3', 2) 5'-CCATGCGAGAGATCACGTCTCCTCGGACG-3', 3) 5'-AGCCGATACTACGTCTCGGAGG-3', 4) 5'-TCCTTCTCTGTACGTCTCGGAGG-3', 5) 5'-CTGTAAGGTAATTCCGCCACG-3', 6) 5'-CTGCGGGAATAACTLCACGGTTACG-3', 7) 5'-CAGGAAACTCTGACCTGTAAGCTTATCCAG-3', 8) 5'-CAGGAAACTCTGACCTGTAAGCTTATCCAG-3', 9) 5'-CAGGAAACTCTGACCTGTAAGCTTATCCAG-3', 10) 5'-CAGGAAACTCTGACCTGTAAGCTTATCCAG-3', 11) 5'-CAGGAAACTCTGACCTGTAAGCTTATCCAG-3', 12) 5'-CAGGAAACTCTGACCTGTAAGCTTATCCAG-3'. The CD5-463X construct was generated using primers 1 and 2, and the BD-CD5-437X construct was generated using primers 7 and 8. Primers 5 and 6 were also used to generate pCDNA3 CD5-384X and primers 7 and 8 were used to generate pCDNA3 CD5-437X. To generate pCDNA3 CD5 Y429F, Y441F, and Y463F (3Y-F), six primers were used as follows: 1) 5'-GAGACTGACATTGATCAGCACAAGTGGCAGTGC-3', 2) 5'-TGGCAATCTACACAGTCTGTCGGCAGTGC-3', 3) 5'-GGGCAATGCTACACAGTCTGTCGGCAGTGC-3', 4) 5'-GGGCAATGCTACACAGTCTGTCGGCAGTGC-3', 5) 5'-GGGCAATGCTACACAGTCTGTCGGCAGTGC-3', 6) 5'-GGGCAATGCTACACAGTCTGTCGGCAGTGC-3'. This construct was made sequentially by generating Y429F, then Y441F, and finally, Y463F. To generate GST CD5 fusion protein, the CD5 cytoplasmic domain DNA was amplified using sense primer 5'-GTGGATCCCGGAAATTCGCCCG-3' and the antisense primer 5'-CTGGGTCAGGGAATTTGCGGCGACGCACGCACCG-3' and cloned in frame into the pGEX-4T-3 vector (Amersham Pharmacia Biotech, Piscataway, NJ). The absence of PCR-introduced artifacts, and the presence of desired nucleotide changes were established by bidirectional nucleotide sequencing using dye terminator chemistry (Applied Biosystems, Foster City, CA). Fusion proteins were prepared using B-PER Bacterial Protein Extraction Reagent (Pierce) as directed by the manufacturer. The fusion proteins were analyzed by SDS-PAGE, and the preparations containing a single band of the appropriate molecular mass were used for subsequent experiments. Protein concentration was quantitated using the Bio-Rad Protein assay (Bio-Rad, Hercules, CA).

Transfections

COS-7 cells (1 × 10^6) were seeded into 100-mm dishes, and 24 h later the cells were transfected with 4 μg of plasmid DNA/dish using the LipoctAMINE Plus kit (Life Technologies) according to the manufacturer’s instructions. For generation of stable transfectants, transfected COS-7 and Jurkat cells were selected using 700 μg/ml and 1 mg/ml Geneticin (Life Technologies, respectively). The transfectants were sorted for equivalent expression after staining with anti-CD5 or anti-CD8 Abs. In some experiments, COS-7 cells were analyzed 48 h following transfection in the absence of any selection (transient transfection).

Internalization assay

To measure CD5 internalization, we used a modified flow cytometric internalization assay (33, 34). Briefly, cells (1 × 10^6/sample) were incubated with PE-conjugated intact anti-human or anti-mouse CD5 mAb (1 μg/ml) for 15 min at 4°C. Following two washes to remove unbound Ab, one set of samples was rapidly warmed to 37°C, while the other was left at 4°C. At different time points (0–30 min), cells were chilled rapidly and all samples were stained with biotin-conjugated anti-human or anti-mouse IgG followed by streptavidin-allophycocyanin staining to detect anti-CD5 mAb remaining on the surface of the cells. All samples were analyzed in a flow cytometer (FACSCalibur, BD Biosciences, Mountain View, CA) to detect PE (FL2) and allophycocyanin (FL4) fluorescence (mean fluorescence intensity; MFI). The percent endocytosis was calculated using MFI values of allophycocyanin (FL4) channel as follows: 100 – [MFI at 37°C/MFI at 4°C] × 100. In experiments that assessed internalization using Fab of CD5 mAb or when stimulation was involved, cells were stimulated for 15 min to remove excess Ab was skipped. The cells were exposed to excess Ab throughout the assay. This was necessary because prebound Fab anti-CD5 dissociated from cell surface at 37°C. For stimulation, cells were treated with anti-CD3 (145-2C11), anti-μ (B76), or Fab(α)'; fragment of goat anti-mouse μ as indicated, followed by the addition of intact anti-CD5 or Fab for an additional 15 min. In some experiments, CD5 internalization was assayed in the presence of brefeldin A (BFA) at 10 μg/ml or cycloheximide (50 μg/ml) as described by Liu et al. (35).

The use of Alexa 488 (FL1) or PE-labeled (FL2) anti-CD5 mAb provided an internal control for loss of Ab from cell surface, since these dyes are detectable within cells even under acidic conditions, as might be expected in lysosomal vesicles. The MFI of total CD5 did not change throughout the treatment period, establishing that decrease in cell surface detected by anti-human or anti-mouse IgG, was a loss of function from cell surface by internalization rather than dissociation from cell surface pool of CD5.
**Immuno precipitation and immuno blotting**

Cells (2 × 10^7) were lysed in 1 ml of lysis buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% v/v Nonidet P-40, 1 mM Na_2VO_4, 10 mM PMSE, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The cell debris was removed by centrifugation. For GST coprecipitation, GST and GST fusion proteins were incubated with lysates, and then precipitated by the addition of glutathione agarose (Amersham Pharmacia Biotech) equilibrated in lysis buffer containing 1 mg/ml of BSA. For coimmunoprecipitation, lysates were incubated with anti-human CD8 (OKT8) followed by precipitation with protein G-agarose. All the precipitates were separated by SDS-PAGE gel electrophoresis. Proteins were transferred onto nitrocellulose membranes and probed with monoclonal anti-α-adaptin (AP-2α) followed by peroxidase-conjugated goat anti-mouse IgG and SuperSignal chemiluminescence substrate. The filters were then stripped (2% SDS, 62.5 mM Tris-HCl (pH 6.8), 100 mM 2-ME) for 30 min at 60°C and reprobed with rabbit antisemur to CD5 cytoplasmic tail (32).

**Results**

**CD5 internalization induced by receptor ligation**

Previous studies have shown that Abs to CD5 induce internalization of CD5 in T and B cell lines and human PBL; however, the mechanism responsible for CD5 internalization has not been defined (36–38). We measured CD5 internalization in Jurkat cells and in COS-7 cells transfected with human CD5 following incubation with a fluorescent-tagged anti-CD5 Ab (Fig. 1A). The kinetics of CD5 internalization in both cell lines showed that endocytosis peaked within 15 min after transfer of cells from 4 to 37°C. To determine whether CD5 is internalized in normal cells, endocytosis was assessed in mature splenic T cells, peritoneal B1a B cells, and splenic B cells from CD5 transgenic mice following incubation with fluorescent-tagged intact anti-mouse CD5 mAb. In each case, CD5 was rapidly internalized, reaching a plateau within 20 min similar to that observed in cell lines (Fig. 1B and data not shown). Internalization of CD5 following incubation with F(ab′)_2 anti-CD5 was comparable to that obtained with intact anti-CD5 (Fig. 1B). To determine whether dimerization or cross-linking of CD5 was necessary to induce internalization of CD5, we generated Fab of anti-CD5. We observed that in both T cells and B cells, monovalent ligation with Fab anti-CD5 was sufficient to induce rapid internalization of CD5 (Fig. 1B). The observed internalization of CD5 was not due to dissociation of anti-CD5 mAb from extracellular CD5 or due to small amounts of aggregated Fab anti-CD5 (see Materials and Methods).

The internalization of CD5 following Ab binding may represent constitutive turnover of CD5 from cell surface rather than ligand-induced endocytosis because our assay does not distinguish between these processes. To directly assess the constitutive turnover rate of CD5, we used BFA that blocks expression of newly synthesized proteins by inhibiting transport of proteins from endoplasmic reticulum (ER) to Golgi and also reexpression (recycling) of endocytosed proteins by causing fusion of early endosomes with the trans-Golgi network (39, 40). The protein synthesis inhibitor, cyclohexamide, was used to distinguish between effects of BFA on ER to Golgi transport and endosomes trans-Golgi network fusion. The transferrin receptor (CD71) is known to be rapidly recycled and its expression was reduced by ~50% within 2 h following BFA treatment (Fig. 2 and Ref. 41). Some of the reduction in surface expression is a result of blockade in transport of newly synthesized protein from ER to Golgi as demonstrated by cyclohexamide treatment. In contrast to transferrin receptor, the turnover rate of CD5 was very slow from both T and B cells (Fig. 2). Treatment of lymphocytes with BFA or cyclohexamide separately or together did not significantly reduce surface expression of CD5 throughout the 4-h time period of the assay, indicating that the cells surface expression of CD5 is relatively stable. The expression of CD45 and Thy1 were slightly reduced by either BFA or cyclohexamide, showing that the turnover rate of these proteins was higher than that of CD5, but still lower than that of CD71, and is consistent with a previous report (35). The rapid loss of CD5 expression from the cell surface after incubation with monovalent and bivalent Ab in the absence of high constitutive turnover demonstrates that CD5 is actively endocytosed following receptor ligation and this process does not require receptor dimerization.

**Effect of BFA on Ab-mediated endocytosis of CD5**

CD5, once internalized, may traffic to lyosomal vesicles for degradation or be recycled back to the surface. If CD5 is recycled following endocytosis, we would predict that loss of surface CD5 following receptor ligation would be much greater in the presence of BFA that also inhibits reexpression of endocytosed proteins. We
observed that decrease in surface CD5 levels in T cells induced by intact anti-CD5, F(ab’2) anti-CD5 or Fab anti-CD5 was similar in the presence or absence of BFA (Fig. 3). In B cells, there was a general trend of greater reduction of CD5 in the presence of BFA and the difference was significant if Fab anti-CD5 was used to induce internalization of CD5. Overall, the results suggest that the majority of CD5 is trafficked to lysosomal degradation pathway rather than recycled back to the cell surface.

The cytoplasmic tail of CD5 associates with the AP2 adaptor complex

The kinetics of CD5 internalization and the ability of monovalent ligands to induce CD5 internalization suggest that CD5 modulation does not result from homeostatic turnover of the plasma membrane but rather is an active response facilitated by an associated molecule. To identify the protein(s) that may be involved in this process, we used the yeast two-hybrid system and screened a human GAL4 activation domain (AD) PBL library with the GAL4 DNA-BD fused to the cytoplasmic tail of CD5 as bait. One cDNA isolated from the screen had a sequence identical with the μ2 subunit (also known as AP50) of the AP2 adaptor complex (42). This cDNA represented 20% of 500 potential CD5 interacting clones (32). None of the AD-μ2 clones sequenced were full-length. The longest cDNA clone represented aa 2–436, and the shortest represented aa 121–436, indicating that the first 121 aa were not necessary for interaction with CD5 (Fig. 4A). To determine the specificity of the interaction, we selected two transmembrane proteins, Igκ and PirB, that contain potential tyrosine-based internalization motifs in their cytoplasmic tail (42). BD-cytoplasmic tail fusion constructs of these proteins were unable to interact with AD-μ2 (clone AD-489; Table I). Similar results were obtained with another AD-μ2, clone AD-500 (data not shown).

The AP2 adaptor complex is a heterotetramer consisting of two large subunits (α2 and β2), an intermediate subunit (μ2), and a small subunit (δ2) (42). None of the subunits exist within cells as free molecules. To determine whether the association of the AP2 adaptor complex with CD5 could be demonstrated in mammalian cells, we performed coimmunoprecipitation experiments. For these studies, we used Jurkat cells that were transfected with chimeric CD8-CD5 protein in which the extracellular region was human CD8 and the transmembrane and cytoplasmic tail are from CD5. Nonidet P-40 lysates from CD8-CD5 expressing and untransfected Jurkat cells were immunoprecipitated with anti-CD8 mAb (OKT8) and Western blots were probed with anti-AP2α mAb (clone 100/5).

FIGURE 3. Effect of BFA on Ab-dependent internalization of CD5. Internalization of CD5 in T and B cells was determined 15 min following incubation with intact Fab’2 of Fab anti-CD5 at 37°C in the presence or absence of BFA (10 μg/ml). Data represent mean ± SD of triplicate values from a representative experiment (n = 3).

The AP2 binding site maps to Y429 in the CD5 cytoplasmic tail

The AP2 adaptor complex binds to internalization motifs in the cytoplasmic tail of transmembrane proteins and mediates recruitment to clathrin-coated pits (42). The internalization sequences are

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Table I. Specificity of interaction between AD-AP50 and BD-CD5

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a Growth at 3 days.
b Clone AD-489.
comprised of either tyrosine-based motifs, NPxY and Yxx/[/L], or di-leucine (LL) motif. CD5 cytoplasmic tail has four tyrosines, three of which have the consensus motif Yxx/[/L], Y378, Y429, and Y441. Nevertheless, Y378 is an unlikely candidate since it represents either the last amino acid of the transmembrane domain, or the first amino acid of the cytoplasmic tail. To determine which of these tyrosines mediate the interaction with $\mu_2$, we generated a panel of truncation and deletion mutations in the CD5 cytoplasmic tail that lack one or more of these motifs and tested them in the yeast two-hybrid assay (Fig. 5A). We found that while the deletion of Y463 did not affect the ability of $\mu_2$ to interact with CD5, in contrast, BD-CD5 constructs that lacked both Y429 and Y441 with unaltered Y378 exhibited no interaction with $\mu_2$. This result indicated that Y429, Y441, or both were involved in the interaction with CD5. To identify which of these tyrosines comprise the binding site of $\mu_2$, we generated a truncated BD-CD5 construct, BD-CD5-437X, which lacked Y441 and tested its ability along with controls to interact with $\mu_2$ in the yeast two-hybrid assay. Based on a liquid lacZ assay, we found that BD-CD5-437X interacted as efficiently as BD-CD5-463X mutant and with slightly lower levels of lacZ than “wild-type” BD-CD5 (Fig. 5B). This observation suggested that the tyrosine most likely to be involved in the interaction with $\mu_2$ was Y429. To test this directly, we generated two additional mutants, BD-CD5Δ429-433 (deletes Y429-P433) and BD-CD5-Y429A. Both of these constructs when cotransformed with AD-$\mu$2 (clone AD489) into yeast cells exhibited very low lacZ activity comparable to that obtained BD-CD5-384X, which lacks most of the cytoplasmic tail (Fig. 5B). The yeast two-hybrid data correlated with the ability to coprecipitate AP2$\alpha$ from Jurkat cell lysates with wild-type, but not Y429A GST fusion proteins of CD5 cytoplasmic tail (Fig. 4C). Mutation of all three tyrosines to phenylalanine (GST-3Y-F) did not affect the ability to coprecipitate AP2$\alpha$. Overall, these data confirm that Y429 is required for the interaction with the AP2 adaptor complex.

Y429 is associated with ligand-dependent and -independent CD5 internalization

Mapping studies using the yeast two-hybrid assay established that Y429 was necessary for the interaction with $\mu_2$. To determine whether this interaction is important for CD5 internalization, we generated a panel of human CD5 mutant constructs in which the Y429 codon was deleted or substituted with that encoding phenylalanine or alanine. We transiently transfected wild-type and mutant CD5 encoding constructs into COS-7 cells and measured their ability to be internalized following ligation with anti-CD5 mAb (Fig. 6). Within 15 min, 40% of wild-type CD5 had been internalized. In contrast, the internalization was inhibited by ~70% in CD5 mutants that lack Y429 (CD5Δ429-433 and CD5Δ384X). Mutation of all three tyrosines in the cytoplasmic tail of CD5 to phenylalanine (CD5–3Y-F) did not alter CD5 internalization, but mutation of Y429 to alanine dramatically inhibited CD5 endocytosis (Fig. 6). The ~10–12% internalization observed with CD5 constructs containing Y429A mutation or lacking Y429 occurred rapidly (5 min) and remained at the same level up to 30 min (data not shown).

The cell surface expression of CD5 on COS-7 cells transiently transfected with constructs in which Y429 was mutated to alanine or deleted was consistently higher than those transfected with wild-type CD5 (data not shown). This result was confirmed by transfection with different clones containing the same mutation and/or different preparations of DNA. These data suggested to us that the AP2 adaptor complex and Y429 may also be involved in ligand-independent down modulation of CD5, thereby regulating steady state expression of cell surface CD5. To directly test this possibility, COS-7 cells were transiently transfected with wild-type and CD5Y429A constructs and 48 h later, the constitutive turnover rate of CD5 was determined using BFA and cyclohexamide. The surface expression of wild-type CD5 was slightly reduced (9%) within 30 min, and by 1 h the level of expression was 77.5% of BFA untreated cells (Fig. 7). In contrast, BFA treatment resulted in only slight decrease in surface expression of Y429A mutant CD5 over the 2-h period of the assay. The loss of surface expression of wild-type CD5 was largely a result of blockade in recycling of CD5 since cyclohexamide treatment only marginally reduced surface CD5 expression. The kinetics of CD5 reduction from cell surface following cyclohexamide treatment was similar for wild-type and Y429A CD5 protein, indicating that the half lives of both these proteins were similar and independent of AP2 binding (Fig. 7). Based on these data, we conclude that the AP2 binding site, Y429, is necessary for ligand-dependent and -independent CD5 internalization.

Effect of TCR or BCR cross-linking on CD5 internalization

Previous studies showed that the $\mu_2$ subunit of the AP2 adaptor complex is unable to bind to tyrosine-based internalization motifs in which the critical tyrosine is phosphorylated (43–45). Those

![FIGURE 5](http://www.jimmunol.org/services.cfm?Service=ViewImage&ImageId=35100380)

**FIGURE 5.** Mapping of AP2 $\mu_2$ binding site on CD5. A. Wild-type BD-CD5 and deletion/truncation constructs were screened for interaction with AD-$\mu_2$ clone, AD489, in the yeast two-hybrid assay for growth on histidine-deficient plates. B. Wild-type BD-CD5 and deletion/truncation/substitution constructs were screened for interaction with AD-$\mu_2$ clone, AD489, in a yeast two-hybrid assay for lacZ activity. Data represent mean ± SD of triplicate values from a representative experiment (n = 3).

![FIGURE 6](http://www.jimmunol.org/services.cfm?Service=ViewImage&ImageId=35100381)

**FIGURE 6.** Tyr$^{429}$ is necessary for CD5 internalization. CD5 internalization was measured in COS-7 cells transiently transfected with constructs that encode wild-type or deletion/substitution mutants of CD5. Internalization was assessed 15 min after incubation with intact anti-CD5 at 37°C. Data represent mean ± SD of triplicate transfections from a representative experiment (n = 3).
biochemical and cell biological studies were confirmed when the crystal structure of the μ2 subunit bound to peptide was solved showing that the substrate-binding pocket of the μ2 subunit lacks the space for binding of tyrosine-based internalization motifs in which the tyrosine is phosphorylated (46). Ag receptor cross-linking in both B and T cells induces rapid tyrosine phosphorylation of CD5 (22, 24). Therefore, we tested the possibility that Ag receptor stimulation would ablate CD5 internalization by blocking the ability of the AP2 adaptor complex to interact with it. For these experiments, we used normal T cells from mouse spleens, a mouse B cell line, A20, which expresses relatively high levels of CD5, and a mouse T cell line, EL4. B cells were obtained from spleens of CD5 transgenic mice in which CD5 is constitutively and selectively expressed on all B cells at levels equivalent to that expressed on endogenous B1a B cells (20). Cross-linking of the BCR on splenic B cells with intact anti-μ Ab completely inhibited CD5 internalization measured using Fab anti-CD5 mAb (Fig. 8). In contrast, CD3 stimulation of splenic T cells with anti-CD3 mAb (145-2C11) consistently augmented CD5 internalization. The apparent dichotomy in the effect of Ag receptor stimulation on CD5 internalization between T and B cells was also reproduced in the A20 B cell line and the EL4 T cell line (Fig. 8). In fact, cross-linking of the Ag receptor on A20 B cells with anti-IgG resulted in 18.3% increase in net expression of surface CD5 within 15 min, most likely from an endogenous presynthesized pool (Fig. 8). Similar results were also obtained if intact anti-CD5 mAb was used to assess internalization, indicating that the effect of Ag receptor stimulation on CD5 endocytosis was independent of CD5 cross-linking.

Use of intact Abs to stimulate the BCR induces coaggregation of the low affinity FcR, FcyRIIB1, and delivery of an inhibitory signal (47). To determine whether signals through the FcR might be responsible for the inhibition of CD5 internalization following BCR stimulation with intact Abs to IgM or IgG, we used the F(ab′)2 fraction of Abs to stimulate CD5+ B cells and the A20 cell line. We found that stimulation of B cells with F(ab′)2 anti-μ or A20 cells with F(ab′)2 anti-IgG resulted in enhanced internalization paralleling that observed in T cells following CD3 stimulation (Fig. 8). To directly test whether FcRs on B cells were responsible for the inhibition of CD5 internalization following BCR stimulation, we used the FcR-deficient A20 B cell line, IIAI6. The kinetics of CD5 internalization were assessed following incubation with intact anti-CD5 mAb in the presence or absence of BCR stimulation with intact Abs to IgG. CD5 was rapidly internalized in A20 and IIAI6 cells reaching a plateau at 15 min for both cell lines (Fig. 9). However, in the presence of BCR stimulation, CD5 internalization was completely inhibited in A20 cells, but not in IIAI6 cells in which a slight increase was noted. Even though IIAI6 cells exhibited similar kinetics of CD5 internalization when compared with A20 cells, the amount of internalization was greater in IIAI6. The difference in net internalization is most likely due to intrinsic differences between these two cell lines. We observed no difference whether we used F(ab′)2 anti-CD5 instead of intact anti-CD5 to induce CD5 internalization, indicating that the Fc portion of anti-CD5 did not stimulate the FcR (data not shown). Overall, these results show that signals through either the BCR or TCR can modulate CD5 internalization. In B cells, depending on whether FcRs are coaggregated or not, the net effect can be inhibition or enhancement of CD5 endocytosis.

**Discussion**

In this study, we have defined the mechanism of CD5 internalization and demonstrated that it can be differentially regulated in T and B cells. Endocytosis of CD5 occurred rapidly in response to monovalent or bivalent Ab ligation in cell lines expressing either endogenous or transfected CD5, in normal mature T cells, and in mature primary B cells that express CD5 endogenously (peritoneal B1a B cells) or induced as in CD5-transgenic mice. We determined, using BFA and cyclohexamide, that CD5, unlike the TCR: CD3 complex (35) exhibits a low constitutive rate of turnover; and therefore, the rapid internalization of CD5 in response to receptor dimerization or cross-linking primarily represents ligand-induced endocytosis. Using BFA, we were also able to show that in both normal T and B cells, CD5 was not recycled back to surface following endocytosis with reagents that would dimerize the receptor. However, if endocytosis was initiated with monomeric Ab, ~10% of CD5 was recycled back to the surface in B cells, but none in T cells.
In an effort to define the mechanism responsible for CD5 endocytosis, we determined that the AP2 adaptor complex directly associates with the cytoplasmic tail of CD5 via its $\mu_2$ subunit. Because the AP2 adaptor complex and clathrin triskelions form the major structural components of plasma membrane-coated pits and vesicles, our results indicate that CD5 internalization is mediated via the clathrin-coated pit-dependent pathway. The AP2 adaptor complex is a heterotrimer consisting of four subunits, two large, $\alpha_2$, and $\beta_2$ (110–115 kDa), a medium, $\mu_2$ (50 kDa) and a small, $\delta_2$ (17 kDa). Of the four recognized endocytic signals (48), the tyrosine-based motifs, NPxy or Yxylx(+)lx (42), and the dileucine motif, LL, interact with AP2, and only the Yxylx(+)lx motif binds to the $\mu_2$ subunit (46, 48). The cytoplasmic tail of CD5 has two tyrosines (Y429 and Y441) that lie within an YxxxF motif and we mapped the interaction to Y429 in the motif $^{429}$YxSF$^{432}$. The interaction of $\mu_2$ with CD5 was essentially abrogated when the YxSF motif was deleted or the critical tyrosine was mutated to alanine. The lack of any effect on the ability of $\mu_2$ to bind CD5 in which Y429 was mutated to F can be expected since the FxxF motif can fit into the hydrophobic pocket of $\mu_2$ and has been shown to efficiently bind AP2 (41, 49). The requirement of Y429 for CD5 endocytosis functionally links AP2 to internalization of CD5 via clathrin-coated pits (Fig. 6). The reason for differences between the current study, which demonstrates a requirement for Y429 for CD5 endocytosis and a previous report showing that the region which includes this amino acid was not necessary for internalization is not completely clear (50). In this study, we assessed the mechanism of rapid down modulation (within first 30 min) of CD5 whereas in the other report internalization was assessed at 3 and 6 h following Ab binding, and it is possible that intermediate vs late internalization of CD5 may be regulated differently. Additionally, in the previous study some of the internalization may reflect changes in surface expression that are related to the half-life of CD5, which is not dependent on Y429 (Fig. 7).

CD5 endocytosis, in addition to being mediated via clathrin-coated pits, is also dependent on interaction with the cytoskeleton. Reorganization of the cytoskeleton and polymerization of actin was shown to be required for CD5 endocytosis (37). In fact, the L chain complex of the dynein complex, Tctex-1, associates with the membrane proximal region of CD5 (51). The H chains of the dynein complex interact with the cytoskeleton, whereas the L chains are involved in linking transmembrane proteins to membrane transport vesicles.

The Ab-dependent endocytosis, especially Fab anti-CD5-mediated endocytosis, is likely to represent ligand-dependent endocytosis under physiological conditions. Though the clear identity of the CD5 ligand has not been established, a good candidate is the recently identified 150-kDa protein that occurs as both membrane-associated form on monocytes and lymphocytes, and as a proteolytically cleaved soluble protein (10). In addition to ligand-dependent endocytosis, the AP2 adaptor complex also seems to be involved in regulating the constitutive turnover of CD5 by a mechanism that is ligand-independent as observed for CTLA4 (Refs. 43–45; Fig. 7). Physiologically, this is likely to be very important for lymphocyte homeostasis because elevated CD5 expression is associated with inhibiting induction of B cell tolerance in immature B cells and colocalization in mature peripheral B cells (Ref. 52; X. Liang, H. Zhao, and C. Raman, manuscript in preparation). In T cells, CD5 overexpression may lead to depressed negative selection in the thymus allowing for the selection of autoreactive T cell clones since loss of CD5 expression results in dramatically enhanced negative selection (11). Unlike in primary cells, some of CD5 in cell lines is recycled back to the surface after endocytosis, and this may reflect differences between transformed cells and normal lymphocytes.

The finding that Ag receptor cross-linking on both B and T cells enhanced CD5 endocytosis was unexpected. This is because Y429 is a major site of Ag-receptor-induced phosphorylation of CD5 (53) and tyrosine phosphorylation of the internalization motif is expected to preclude interaction with $\mu_2$ (43–45, 54). The lack of inhibition may in part be due to rapid dephosphorylation of Y429 and the fact that not all of the available CD5 is phosphorylated following Ag receptor stimulation. We have not completely resolved the mechanism underlying enhanced endocytosis of CD5 following Ag receptor stimulation in the absence of FcR coligation. A possible mechanism is via the Ag receptor-induced activation of phosphatidylinositol 3-kinase (PI3-kinase) that will catalyze the conversion of phosphatidylinositol 4,5-biphosphate to phosphatidylinositol 3,4,5-triphosphate (PI3,4,5P3). The plekstrin homology (PH) domain in the $\alpha_2$ subunit of the AP2 adaptor complex binds with high affinity to PI3,4,5P3, and localizes AP2 complex to the membrane (55, 56). This interaction also mediates an increase in the affinity of $\mu_2$ binding to tyrosine-motifs to facilitate enhanced receptor endocytosis. Preliminary experiments using the pharmacological agent LY294002, a PI3-kinase inhibitor, indicated that PI3 kinase is involved in the Ag receptor-induced enhancement of CD5 internalization (our unpublished observation).

The inhibition of CD5 internalization following cross-linking of BCR with intact Abs may be a function of Src homology 2 domain-containing 5’ inositol phosphatase (SHIP) activity (Fig. 8). Stimulation of BCR with intact anti-receptor Abs coengages the inhibitory FcγRIIB, resulting in tyrosine phosphorylation of its cytoplasmic tail and recruitment of SHP (47). The involvement of FcγRIIB in inhibition of CD5 endocytosis following BCR stimulation was confirmed by the lack of inhibition of CD5 internalization in FcγRIIB-deficient IIA1.6 cells and when F(ab’)$_2$ Abs were used to stimulate BCR on normal B cells or A20 cells. Because the $\alpha_2$ subunit of the AP2 adaptor complex is localized to the membrane by its interaction with PI3,4,5P3, the catalysis of PI3,4,5P3 to phosphatidylinositol 3,4-diphosphate (PI3,4P2) by SHIP will inhibit the translocation of AP2 to membrane. Consistent with this hypothesis, studies have demonstrated that mutation of the phosphoinositide binding site on AP2 blocks PI3,4,5P3-mediated endocytosis, demonstrating an essential role for PI3,4,5P3 in endocytosis (57).

The ability to differentially regulate CD5 internalization in T and B cells could allow for different net effects of CD5 signaling that may be cell type, developmental stage, and/or activation stage specific. In fact, our recent in vivo studies indicate that CD5 has a net negative regulatory role in immature and mature T cells, whereas in B cells, the net effect of CD5 is inhibitory in immature B cells but can be either costimulatory or inhibitory in mature B cells. The lack of clear knowledge as to how CD5 regulates Ag receptor signaling in normal cells makes it difficult to predict the role of CD5 internalization in this process. One possibility is that the interaction of AP2 with CD5 is required for localizing CD5 into sphingolipid-rich microdomains or rafts in lymphocytes, as has been recently reported (58). Therefore, the inhibition of CD5 internalization by SHIP would inhibit its recruitment into lipid rafts and result in a net positive effect on Ag receptor signaling. Conversely, the enhancement of AP2 association with membrane lipids would facilitate greater recruitment of CD5 into rafts, resulting in greater attenuation of TCR/BCR signaling. It is also possible that CD5 functions as a “sink” either to colocalize or segregate activation/inhibitory molecules with respect to TCR/BCR complex. In such a situation, the rapid removal of CD5 from the cell surface or its retention could have opposite effects on Ag receptor signaling. In summary, the ability to posttranslationally regulate the cell surface CD5 expression allows for this receptor to...
function with opposite effects on TCR/BCR signaling without the need to invoke a distinct set of signaling pathways for different functions. This property may also explain why the published literature often conflicts as to the physiological role of CD5 in regulation of Ag receptor signaling.

The mechanism by which AP2-mediated CD5 internalization is regulated is likely to be important because CD5 plays a fundamental and important role in B and T lymphocyte selection, activation, and generation of tolerance. In addition to SHIP described above, another key regulator of AP2-mediated endocytosis may be CK2, an important regulator of receptor endocytosis (59). We and others have shown that CK2 constitutively associates with and is activated by CD5 (32, 60). The development of transgenic mice that express CD5 on B and/or T lymphocytes lacking the ability to interact with the AP2 adaptor complex will be key in defining the physiological role of posttranslational regulation of CD5 expression.

Acknowledgments

We thank Dr. Robert Kimberly for critical review of the manuscript and Anling Kuo for technical help.

References


