CD5 and CD6 are closely related lymphocyte surface receptors of the scavenger receptor cysteine-rich superfamily, which show highly homologous extracellular regions but little conserved cytoplasmic tails. Both molecules are expressed on the same lymphocyte populations (thymocytes, mature T cells, and B1a cells) and share similar co-stimulatory properties on mature T cells. Although several works have been reported on the molecular associations and the signaling pathway mediated by CD5, very limited information is available for CD6 in this regard. Here we show the physical association of CD5 and CD6 at the cell membrane of lymphocytes, as well as their localization at the immunological synapse. CD5 and CD6 co-immunoprecipitate from Brij 96 but not Nonidet P-40 cell lysates, independently of both the co-expression of other lymphocyte surface receptors and the integrity of CD5 cytoplasmic region. Fluorescence resonance energy transfer analysis, co-capping, and co-modulation experiments demonstrate the physical in vivo association of CD5 and CD6. Analysis of T cell/antigen-presenting cells conjugates shows the accumulation of both molecules at the immunological synapse. These results indicate that CD5 and CD6 are structurally and physically related receptors, which may be functionally linked to provide either similar or complementary accessory signals during T cell activation and/or differentiation.

The correct activation and differentiation of T lymphocytes results from the fine-tuning of intracellular signals delivered through co-engagement of the antigen-specific receptor and a series of accessory molecules simultaneously expressed on the cell surface. Among the lymphocyte accessory molecules (CD2, CD4, CD8, CD9, CD28, CD43, CD45, etc.), there are CD5 and CD6, two highly homologous representatives of the scavenger receptor cysteine-rich (SRCR) superfamily (1, 2). The SRCR superfamily includes a heterogeneous group of soluble and/or membrane-associated receptors involved in the development of the immune system and in the regulation of both innate and adaptive immune responses (1). This family is characterized by the presence of one or several repeats of a well conserved extracellular domain named SRRC, which was first reported at the C terminus of the macrophage type I scavenger receptor (SR-AI) (3). CD5 and CD6 are the two representatives of the SRCR superfamily reported to be expressed on human lymphocytes. Both lymphocyte receptors are type I transmembrane glycoproteins with extracellular regions composed of three SRCR domains and cytoplasmic domains devoid of intrinsic catalytic activity but well adapted for signal transduction (4, 5). The two molecules show a similar cell expression pattern, being expressed on thymocytes, mature T cells, B1a cells, and B chronic lymphocytic leukemia cells (2), and their surface expression levels are also up-regulated by similar stimuli (6, 7). The genes for CD5 and CD6 map to contiguous regions of human chromosome 11q12.2 and are supposed to have arisen from duplication of a common ancestral gene (8, 9).

CD5 has been shown to behave as a dual receptor, which provides either positive or negative co-stimulatory signals depending on the cell type and the developmental stage (10). To achieve this, engagement of different signaling molecules and cascades by CD5 (phosphatidylcholine-specific phospholipase C (PC-PLC), acidic sphingomyelinase (ASMase), casein kinase II (CKII), and phosphatidylinositol 3-kinase (PI3K)) and phosphatidylinositol 3-kinase/mitogen-activated protein kinase, etc.) have been reported (10). Initial positive co-stimulatory properties on peripheral T cells were first reported for CD5, by using either soluble or solid phase-bound monoclonal antibodies (mAbs) (11–14). Later, data from CD5-deficient mice have shown that CD5 may also negatively regulate the signaling through the antigen-specific receptor complex on thymocytes and B1a cells (15, 16). Subsequent studies (17–19) have highlighted the importance of CD5 in thymocyte develop-
CD5 and CD6 Expression Constructions—The cDNA used to amplify the cytoplasmic region of CD6 was obtained by retrotranscription of total mRNA from PBL with Superscript™ II RNase H - Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions (Pierce). Mowiol 4-88 was from Calbiochem, and poly-l-lysine (PLL) was from Sigma. The blue fluorescent cell tracker chloromethyl derivative of aminocoumarin (CMAC) was from Molecular Probes (Eugene, OR). Staphylococcal enterotoxin E (SEE) was from Toxin Technology ( Sarasota, FL).

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Antibodies and Reagents—The mouse Cris-1 (anti-CD5, IgG2a), 148.1C3 (anti-CD43, IgG2a), 33-2A3 (anti-CD3, IgG2a) and 161.8 (anti-CD6, IgG1) mAbs were produced in our laboratory by R. Vililla (Hospital Clinic, Barcelona, Spain). Affinity-purified Leu-1 (anti-CD5, IgG2a) mAb was purchased from BD Biosciences. The SPV L14.2 (anti-CD6, IgG1) mAb was from Immunotech (Marseille, France), and the W6/32 (anti-FLAG class I, IgG2a) was from the ATCC (HB-95). The fluorescein isothiocyanate (FITC)-labeled anti-CD5 (UCHT1, IgG, anti-CD6 (M-T605, IgG), and anti-CD3 (UCHT1, IgG) mAbs were from Pharmingen. The 141.1C5 mAb was conjugated to FITC (Sigma) as described previously (45). The Leu-1 and 161.8 mAbs were conjugated to cyanine 3 (Cy3) using the Cy3 mAb labeling kit (Amersham Biosciences). The FITC-conjugated goat anti-mouse polyvalent immunoglobulins (GAMig-FITC) were from Sigma. Horseradish peroxidase (HRP)-conjugated streptavidin (SAv) was from Duko (Denmark). Trifluorothymidine (CFT; Pharmacia, Uppsala, Sweden) and puromycin (Calbiochem) were purchased from Sigma. 148.1C3 mAb was conjugated to FITC (Sigma) as described previously (45). The Leu-1 and 161.8 mAbs were conjugated to cyanine 3 (Cy3) using the Cy3 mAb labeling kit (Amersham Biosciences). The FITC-conjugated goat anti-mouse polyvalent immunoglobulins (GAMig-FITC) were from Sigma. Horseradish peroxidase (HRP)-conjugated streptavidin (SAv) was from Duko (Denmark). Trifluorothymidine (CFT; Pharmacia, Uppsala, Sweden) and puromycin (Calbiochem) were purchased from Sigma.

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FRET = FRET - (A × FITC) - (B × Cy3)  

\[ \text{FRET} = \frac{E_F - E_C}{E_F} \]

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where FRET and Cy3 are the mean intensities of FRET and Cy3 in the selected regions of interest. These calculations allowed the E_F to be < 0. All calculations were performed using the Image Processing Leica confocal software and Microsoft Excel. The statistical analysis was performed by SPSS statistical software (Chicago, IL). The results are graphed showing the mean ± S.D. and percentiles 25 and 75. Statistical differences between groups were tested using the non-parametric Mann-Whitney test. A value of \( p < 0.001 \) was taken to indicate statistical significance.

**Fluorescence Analysis of Cell Conjugates—** T-cell-APC cell conjugates were generated by using Vβ8 TCR-expressing Jurkat cells (J77c120) and the human B cell line Raji in the presence or absence of SEE as described previously (52). Jurkat J77 cells were loaded with 10 μM CMAC for 20 min at 37°C. Raji cells (5 × 10^6 cells/ml) were resuspended in Hanks' balanced salt solution and incubated for 20 min in the presence or absence of 5 μg/ml SEE. The Raji cells (5 × 10^6 cells) were then mixed with an equal number of Raji cells in a final volume of 600 μl and incubated for 30 min before plating onto PLL-coated slides in flat-bottomed 24-well plates (Costar Corp.). Cells were allowed to settle for 10 min at 37°C, fixed for 5 min in PBS 2% formaldehyde, and blocked with 100 μg/ml human IgG (Sigma) before staining with the appropriate mAbs plus FITC-GAM Ig. Cells were observed by a DMR photomicroscope (Leica) with ×63 and 100 oil immersion objectives. Images were acquired using the Leica QFISH 1.0 software. Conjugates were first identified by directly observing both cell morphologies under differential interference contrast and blue fluorescent CMAC-labeled J77 cells.

**RESULTS**

The CD5 and CD6 Surface Receptors Co-precipitate in Normal and Leukemic Human T Lymphocytes—The possible membrane association of CD5 and CD6 was explored by co-immunoprecipitation experiments of biotin-labeled surface proteins solubilized under different detergent conditions (either 1% Brij 96 or 1% Nonidet P-40). The presence of CD6 in CD5 immunoprecipitates was investigated by re-precipitation with a CD6-specific rabbit polyclonal antisera. Conversely, a similar re-precipitation procedure was used to investigate the presence of CD5 in CD6 immunoprecipitates. Experiments performed with the human leukemic T cell line HUT-78 showed 105–130-kDa bands in CD5 immunoprecipitates from Brij 96 but not Nonidet P-40 lysates (Fig. 1A). No similar bands were detected after CD6 re-precipitation of HLA class I and CD3 immunoprecipitates from Brij 96 lysates of biotin-labeled HUT-78 cells (Fig. 1B). The bands observed agreed with the reported molecular mass of CD6, for which an un-phosphorylated 105-kDa form exists that rapidly changes to a phosphorylated 130-kDa form by exposure to serum or activators of protein kinase C (29, 33). Conversely, a 67-kDa band, probably corresponding to CD5, was observed after re-precipitating CD6 immunoprecipitates with a CD6-specific polyclonal antisera (Fig. 1A). Depletion experiments showed that removal of either CD5 or CD6 by sequential immunoprecipitations abrogated the co-precipitation of the two molecules (Fig. 1C), thus confirming that the 67 and 105–130 kDa bands detected by our polyclonal antisera are specific and almost certainly correspond to CD5 and CD6, respectively. Further experiments performed with surface-biotinylated thymocytes and lymph node cells (Fig. 1D) demonstrated the association of CD5 and CD6 on normal cell types.
The Association of CD5 and CD6 Is Independent of Co-expression of Other Lymphocyte Surface Receptors and of the Integrity of the CD5 Intracellular Region—CD5 is known to associate with other lymphocyte receptors (CD2, CD3, CD4, CD8, and CD9) under Brij 96-mediated cell membrane solubilization conditions (23, 43, 44). Therefore, we used a heterologous cell environment to investigate whether CD5 and CD6 co-precipitation was either direct or mediated by an interposed lymphocyte surface molecule. For this purpose, COS-7 cells were transiently transfected with expression constructs for wild-type CD6 (CD6.WT) and CD5 (CD5.WT), and a tail-less CD5 form (CD5.K378Stop), either alone or in combination. Transfected cells were surface-biotinylated, lysed with 1% Brij 96, and subjected to CD5 immunoprecipitation (Cris-1 mAb). Immune complexes were eluted and then re-precipitated with polyclonal rabbit anti-CD6 serum. Detection of biotinylated proteins was done by HRP-SAv and enhanced chemiluminescence after running in 8% SDS-PAGE under reducing conditions and subsequent transfer to nitrocellulose membranes.

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Measurement of CD6 and CD5 Association by FRET—To circumvent possible detergent-based artifacts, FRET microscopy analysis, a method that can detect molecular proximity between two proteins with a resolution of 10s of Angstroms, was performed on living lymphocytes (51). We utilized Cy3-labeled anti-CD5 mAb as acceptor and a series of FITC-conjugated mAbs against CD6, CD3, and CD43 as donor. The FRET images obtained for the different pairs of mAbs used are shown in Fig. 3A. Positive FRET between FITC-anti-CD6 and Cy3-anti-CD5 mAbs was found, thus confirming the association of CD5 and CD6.
Physical Linkage between CD5 and CD6

Asterisk indicates statistically significant differences (p < 0.001) as deduced from the Mann-Whitney test. Data between CD5 and CD6 deduced by co-precipitation studies. In agreement with previous reports on the physical association of CD5 and the TCR-CD3 complex on the surface of T lymphocytes (42), we also observed FRET between FITC-anti-CD3 and Cy3-anti-CD5 mAbs. On the contrary, very low FRET was observed when FITC-anti-CD43 and Cy3-anti-CD5 mAbs were used, which agrees with our data showing lack of co-capping between CD5 and CD43 molecules (data not shown).

As a control of optimal energy transfer, we included Cy3-anti-CD5 cross-linked with FITC-GAM Ig. The efficiency of energy transference between FITC and Cy3 fluorochromes measured by calculating the sensitized FRET signal (FRET⁰) on a pixel-by-pixel basis is shown in Fig. 3B. The highest median $E_a$ value was observed by cross-linking Cy3-anti-CD5 with FITC-GAM Ig. The median $E_a$ values obtained for the combination of the Cy3-anti-CD5 plus FITC-anti-CD6 mAbs and Cy3-anti-CD5 plus FITC-anti-CD3 mAbs were similar and reached statistical significance when compared with the combination used as negative control (Cy3-anti-CD5 plus FITC-anti-CD43 mAbs) (Fig. 3B). Taken together, our FRET data indicate that CD5 is in close proximity not only with CD3 but also with CD6. The proportion of CD3 and CD6 molecules that associate with CD5 was estimated as 18 and 12%, respectively, from the numerical data presented in Fig. 3B (assuming 100% association for FITC-GAM Ig/Cy3-αCD5 and background levels for FITC-αCD43/Cy3-αCD5 $E_a$ values). This estimate is in agreement with data reported previously showing that 10–20% of CD5 associates with CD3 in human T lymphocytes (53).

CD5 and CD6 Partially Co-cap and Localize at the Immunological Synapse—To confirm further that CD5 physically associates with CD6 on the lymphocyte surface, we examined the ability of the two molecules to co-cap using double immunofluorescence. Co-capping was explored by incubating cells with biotin-labeled mAbs plus TR-SAv for 30 min at 37°C, followed by fixation and staining with FITC-conjugated mAbs. As shown in Fig. 4A, partial co-capping of CD5 and CD6 was observed independently of the direction explored. On the contrary, no co-capping of CD43 was found with CD6 (Fig. 4A) and CD5 (data not shown). The predicted presence of CD3 molecules on the caps induced with anti-CD5 mAbs (42) was confirmed (Fig. 4A).

Next, we explored whether the down-modulation of the surface expression of one of the antigens (CD5 or CD6) reduced the surface expression of the other. For these purposes, lymphocytes were left overnight at 37°C in the presence or absence of unlabeled specific mAbs cross-linked with FITC-GAM Ig. This resulted in complete loss of the corresponding antigen as assessed by comparing the green fluorescence emission (FL1) of mAb-modulated cells with that of unmodulated cells (data not shown). Then the cells were stained with biotinylated specific mAbs plus Tricolor-SAv and analyzed for red fluorescence (FL3). As shown in Fig. 4B, a reduction in CD5 surface expression of CD6-modulated cells was observed, compared with that of unmodulated cells. Similarly, a reduction in CD6 expression was also observed after mAb-induced modulation of CD5 (Fig. 4B). Taken together, the co-capping and co-modulation results further extend our evidence on the physical association of CD5 and CD6 on the membrane of lymphocytes.

The recruitment of the CD5 and CD6 receptors to the same cap structures might be a nonspecific process related to membrane flow and cell motility. Therefore we decided to investigate the localization of the two molecules on more physiological supramolecular activation clusters (SMACs) formed at the interface of physical contact between T cells and APCs, also known as immunological synapse (IS). APC-T cell conjugates between Raji B cells and J77c120 Jurkat T cells were generated in the presence or absence of superantigen SEE. At 30 min, the localization of CD5 or CD6 at the cell interface was studied by staining with specific mAb plus FITC-GAM Ig. As shown in Fig. 4C, both CD5 and CD6 accumulated at the contact zone between

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**Fig. 3. Detection of CD5 and CD6 association by FRET.** A, PBLs were simultaneously stained with Cy3-conjugated (acceptor) anti-CD5 mAb and FITC-conjugated (donor) antibodies against either CD3, CD6, CD43, or mouse immunoglobulins (GAMIg). FRET⁺ images were obtained as described under "Material and Methods" and presented as pseudocolor intensity-modulated images. alufl, arbitrary linear units of fluorescence intensity. Bar, 2 μm. B, the apparent efficiencies of energy transference between FITC and Cy3, $E_a$, were calculated for several cell membrane regions (regions of interest). The median $E_a$ values ± S.D. are presented and graphed indicating the 25 and 75 percentiles. Asterisk indicates statistically significant differences (p < 0.001) as deduced from the Mann-Whitney test.
J77c120 and Raji cells in an antigen-specific manner. These data indicate that the association of CD5 and CD6 detected in resting lymphocytes is maintained when highly specialized and functionally relevant structures, such as IS, are formed.

DISCUSSION

Lymphocytes express numerous receptors, which continuously engage with ligands on cell surfaces. Some of these receptor/ligand pairs have co-stimulatory or inhibitory capacity. The dynamic cross-talk between these receptors ultimately governs the cell activation state. The present report shows that a fraction of the CD5 and CD6 lymphocyte receptors associate at the membrane of resting cells (thymocytes, lymph node cells, and PBL), likely constituting a functional unit. This is the first reported association for CD6 and provides further evidence on the relationship existing between the physiology of CD5 and CD6 on supramolecular membrane structures.

Fig. 4. Association of CD5 and CD6 on supramolecular membrane structures. A, co-capping of CD5 and CD6. PBLs were induced to cap at 37 °C with biotinylated (b) mAbs plus TR-SAv. At time 0 and 30 min, the cells were washed with ice-cold PBS/azide and stained with the indicated FITC-conjugated mAbs. The images show the red (TR) and green (FITC) fluorescence. B, co-modulation of CD5 and CD6. Lymph node cells were subjected to CD5 or CD6 mAb-induced modulation by overnight incubation at 37 °C plus FITC-GAM Ig. At the end of the incubation period cells were stained with biotinylated (b) mAbs against CD5 or CD6 plus Tricolor-SAv and subjected to flow cytometry analysis. The histograms show the red fluorescence (FL3, in log scale) of modulated (gray line) and unmodulated (black line) cells with anti-CD6 (left) and anti-CD5 (right) mAbs. C, localization of CD5 and CD6 in T cell-APC conjugates. Raji cells were incubated with or without 5 μg/ml of SEE and mixed with Jurkat J77 cells probed with CMAC. After 30 min of incubation, cell conjugates were adhered to PLL-coated coverslips, fixed, and stained (in green) for CD5 and CD6. The corresponding differential interference contrast images were superimposed on the blue staining of J77 cells.
CD5 and CD6, not only at structural but also at functional level. Importantly, this association seems to be maintained following lymphocyte activation because they co-localize when more ordered structures, such as caps, are formed at the lymphocyte membrane. The cap structures consist of the assembly of receptors and signaling molecules involved in lymphocyte activation, which resembles that of SMACs at the interfaces of physical contact between T cells and APCs (54). Although the biological significance of caps is questionable, that of the SMACs is indubitable. It is the TCR-mediated stimulation following contact between TCRs and major histocompatibility complex ligands expressed on APCs that leads to the formation of SMACs (54). Thus, accumulation of CD5 and CD6 at this structure implies a relevant role for these molecules in the signaling processes taking place during T cell activation and differentiation. This is not surprising because both CD5 and CD6 have cytoplasmic tails that are well adapted for signal transduction. Furthermore, co-stimulatory properties on mature T cells have been reported for both molecules. Similarly, the certain role played by CD5 in thymocyte selection is probably shared by CD6, as deduced from the already available evidence (40).

During lymphocyte maturation, developing T cell precursors are in continuous contact with stromal cells of the thymic microenvironment, and this physical contact is crucial for differentiation and selection. In this regard, it is known that both CD5 and CD6 are coordinately expressed from early (pro-T and double negative) stages of thymocyte development (22, 40) and possess ligands expressed on thymic epithelial cells (26, 37, 38). This fact, together with the association data herein reported, may indicate that CD5 and CD6 are also functionally linked to influence thymocyte development and selection. This situation would be reminiscent of that reported previously (24) for CD2 and CD5. Both molecules present a similar pattern of expression during T cell ontogeny (40), have been reported to be associated independently of CD3 (23), and have synergistic effects on thymocyte-positive selection (24). Therefore, the availability in the future of single (CD6) and double (CD5/CD6)-deficient mice would help to confirm the possible functional linkage existing between CD5 and CD6 during intrathymic selection and to demonstrate whether they are connected with a common signaling pathway. Thus, if confirmed, it might indicate that redundant functional units (CD5/CD2 and CD5/CD6) are used to securely drive the thymocyte selection process. This could explain why the extracellular region of CD5 is dispensable for the CD5-mediated down-regulation of TCR signaling during thymocyte development (25). Although intact, the extracellular regions of CD2 and/or CD6 would interact with their respective ligands at the thymus and co-engage signals delivered through the associated CD5 molecules. In fact the deletion of the extracellular region of CD6 does not abrogate its association with CD2 (55).

The data presented here indicate that the association of CD5 and CD6 is independent of both the co-expression of other lymphocyte receptors and most of the cytoplasmic region of CD5. These results, however, do not completely rule out the existence of interactions mediated through the cytoplasmic region, and further experiments are needed to fully map the CD5 and CD6 regions involved in this association. Interestingly, it has been reported recently (55) that the association of CD2 with CD5 is held at both the intra- and extracellular levels, and this could also be true in the association of CD5 and CD6. Our results also do not preclude that CD6 could associate independently with other lymphocyte surface receptors. This is illustrated by CD5, which has been reported to associate independently with CD2 and CD3 (23) and, herein, with CD6. The composition and organization of the plasma membrane is not homogenous, and these independent associations could take place at different membrane compartments, such as lipid rafts (56). Indeed, it has been reported that a fraction of CD5 molecules resides in T cell rafts (57). Our preliminary data, however, indicate that pretreatment of lymphocytes with methyl-β-cyclodextrin, which disrupts lipid rafts microdomains, does not abrogate the association of CD5 with CD6. In conclusion, this association of CD5 and CD6 suggests that both molecules may act coordinately during activation and/or differentiation of T lymphocytes by providing either similar or complementary intracellular signals. CD5 and CD6 share very similar extracellular regions but little homologous intracellular regions. Therefore, it can be hypothesized that they could recruit different signaling elements. The two molecules are rapidly phosphorylated on serine/threonine and tyrosine residues following engagement of the TCR-CD3 complex and then may interact with different intracytoplasmic molecules. Although not completely understood, the CD5 signaling pathway has been extensively studied (10). On the contrary, there is still limited information available on CD6-mediated signaling. Thus, in light of the present report, it would be interesting to study not only the signaling capabilities of CD6 but also the possible cross-talk mechanisms existing between CD5 and CD6.

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The Accessory Molecules CD5 and CD6 Associate on the Membrane of Lymphoid T Cells

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