

# Differential Regulation and Function of CD73, a Glycosyl-Phosphatidylinositol-linked 70-kD Adhesion Molecule, on Lymphocytes and Endothelial Cells

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**Abstract.** CD73, otherwise known as ecto-5'-nucleotidase, is a glycosyl-phosphatidylinositol-linked 70-kD molecule expressed on different cell types, including vascular endothelial cells (EC) and certain subtypes of lymphocytes. There is strong evidence for lymphocyte CD73 having a role in several immunological phenomena such as lymphocyte activation, proliferation, and adhesion to endothelium, but the physiological role of CD73 in other cell types is less clear. To compare the biological characteristics of CD73 in different cell types, we have studied the structure, function, and surface modulation of CD73 on lymphocytes and EC. CD73 molecules on lymphocytes are shed from the cell surface as a consequence of triggering with an anti-CD73 mAb, mimicking ligand binding. In contrast, triggering of endothelial CD73 does not have any effect on

its expression. Lymphocyte CD73 is susceptible to phosphatidylinositol phospholipase, whereas only a small portion of CD73 on EC could be removed by this enzyme. Furthermore, CD73 on EC was unable to deliver a tyrosine phosphorylation inducing signal upon mAb triggering, whereas triggering of lymphocyte CD73 can induce tyrosine phosphorylation. Despite the functional differences, CD73 molecules on lymphocytes and EC were practically identical structurally, when studied at the protein, mRNA, and cDNA level. Thus, CD73 is an interesting example of a molecule which lacks structural variants but yet has a wide diversity of biological functions. We suggest that the ligand-induced shedding of lymphocyte CD73 represents an important and novel means of controlling lymphocyte-EC interactions.

LYMPHOCYTE recirculation is known to be finely regulated by different adhesion molecules expressed on lymphocytes and endothelial cells (EC)<sup>1</sup> and by chemokines, controlling the activation status of the cells (30). Lymphocytes make the initial contact with EC by rolling along the vessel wall in the high endothelial venules of lymphoid tissues or along the flat-walled endothelium at sites of inflammation. Lymphocyte rolling is a well characterized phenomenon, and it is known to be mediated by selectins, situated on the tips of microvilli both on the lymphocyte and EC surface (42). More stable adhesion is achieved through binding of activated integrins to their EC counterparts, and lymphocytes eventually migrate through the vessel wall using integrins and integrin ligands of the Ig superfamily (8, 30).

CD73/ecto-5'-nucleotidase (ecto-5'-NT), is a 70-kD glycosyl-phosphatidylinositol (GPI)-linked molecule which can be detected in several different mammalian tissues and cell types (46). Ecto-5'-NT enzyme activity catalyzes the extracellular dephosphorylation of nucleoside monophosphates to their corresponding nucleosides. This enables the uptake of adenosine, inosine, and guanosine into the cell and their subsequent reconversion into ATP and GTP in the purine salvage pathway (36). The physiological role of ecto-5'-NT, however, probably differs in various organisms and tissues, and it most likely extends beyond its enzymatic activity (46). Plasma membrane-bound ecto-5'-NT (CD73) has been shown to be involved in controlling lymphocyte-EC interactions, as binding of lymphocytes to cultured EC can be inhibited by an anti-CD73 mAb (2, 3). Ecto-5'-NT has also been implicated in cell-matrix interactions in chicken fibroblasts (33) and as a signal transducing molecule in the human immune system (9, 24, 37). In particular, its role as a costimulatory molecule in T cell activation has been well established (14, 24). Transient expression of CD73 on neuronal cells has been described during developmental processes and, on lymphocytes, CD73 serves as a maturation marker, being absent from the surface of both immature B and T cells (15, 37).

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1. *Abbreviations used in this paper:* EC, vascular endothelial cells; ecto-5'-NT, CD73/ecto-5'-nucleotidase; GPI, glycosyl-phosphatidylinositol; HUVEC, human umbilical vein endothelial cells; IF, immunofluorescence; MFI, mean fluorescence intensity; PBL, peripheral blood lymphocytes; PI-PLC, phosphatidylinositol-specific phospholipase C.

A subpopulation of peripheral blood lymphocytes (PBL) expresses CD73 on the majority of B cells and CD8<sup>+</sup> T cells but on only about 10% of CD4<sup>+</sup> T cells (10, 38, 40). CD73/ecto-5'-NT has also been detected in nervous tissue: on venules in various tissues and on follicular dendritic cells in the secondary lymphoid tissue (2, 10, 40). The subcellular expression of ecto-5'-NT has been studied intensively in rat liver tissue where the molecule is expressed both intracellularly and on the surface of hepatocytes. A similar localization has been observed in rat fibroblasts, guinea pig neutrophils, and capillary EC (18, 23, 28, 45, 46). Continuous recycling of ecto-5'-NT between the cell surface and the intracellular pools has been described in hepatocytes, fibroblasts, and rat hepatoma cells (31, 41, 45). The natural ligand(s) of CD73 are not known at present.

As the diversity of ecto-5'-NT-expressing cells and tissues is substantial and the molecule has several putative roles, it is important to clarify its functional and structural properties on different cell types. Thus, we studied properties of CD73 expressed on both cell types having importance in lymphocyte homing, PBL and vascular EC. Significant differences were found in the function and cell surface modulation of CD73 on these cell types.

## Materials and Methods

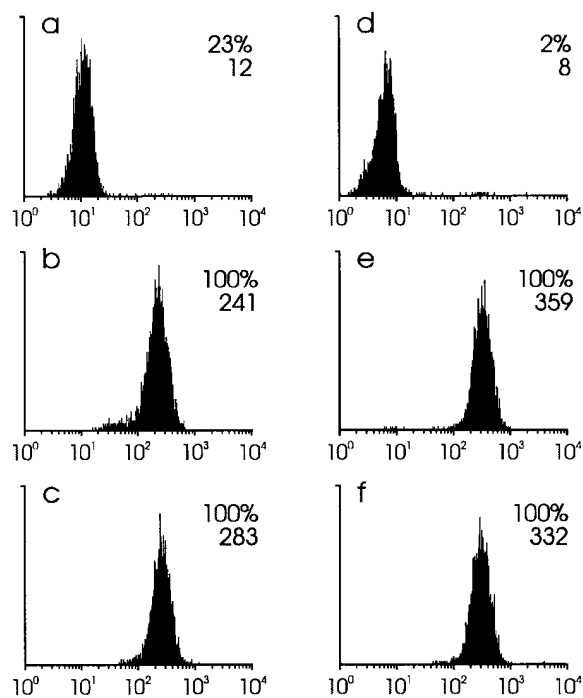
### Cells, Cell Lines, and Antibodies

HUVECs (human umbilical vein endothelial cells) were isolated as described earlier (2). They were cultured on gelatin-coated flasks in EC basal medium (Clonetics Corp., San Diego, CA) supplemented with 10% human AB-serum (Finnish Red Cross, Helsinki, Finland), 50 µg/ml EC growth factor (Boehringer Mannheim GmbH, Mannheim, Germany), 5 U/ml heparin (Sigma Chemical Co., St. Louis, MO), 4 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. HEC-endothelial cell line, equivalent to EaHy-926 cell line (11), was a kind gift of Dr. H. Holt-höfer (University of Helsinki, Finland). HECs were cultured in RPMI-1640 medium supplemented with 10% FCS, 4 mM L-glutamine, and antibiotics (referred to as complete medium). Human PBL from healthy, voluntary donors were isolated using Ficoll-Hypaque (Histopaque-1077; Pharmacia Fine Chemicals, Uppsala, Sweden). For tyrosine phosphorylation studies, peripheral blood mononuclear cells were enriched for CD73<sup>+</sup> cells by depleting CD4<sup>+</sup> and CD11b<sup>+</sup> cells using anti-CD4 (CRL8002, mouse IgG<sub>1</sub>, ATCC) and anti-CD11b (ATCC) antibodies and Dynabeads (Dyna, Inc., Oslo, Norway).

mAb 4G4 (mouse IgG<sub>1</sub>) recognizes CD73 (2, 3). The CD73 workshop mAb 1E9 (mouse IgG<sub>3</sub>) was a kind gift of Dr. Linda Thompson (Oklahoma Medical Research Foundation, Oklahoma City, OK). Binding inhibition studies demonstrated that the 4G4 and 1E9 mAbs recognize distinct epitopes of the CD73 molecule (Fig. 1). For epitope mapping, HEC cells were sequentially incubated with 4G4, 1E9, and an FITC anti-mouse IgG<sub>3</sub> (Southern Biotechnology, Birmingham, AL) or with 1E9, 4G4, and an FITC anti-mouse IgG<sub>1</sub> (Southern Biotechnology) and analyzed by FACScan<sup>®</sup>. mAb Hermes-3 recognizes CD44 (16). Anti-phosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). An irrelevant mAb against chicken T cells (3G6) was used as a negative control.

### mAb Treatment of Lymphocytes and HEC

Cells were treated in the following four different ways: (a) lymphocytes and EC were resuspended in 3G6, 4G4, or 1E9 mAb (either at 37 or 4°C) containing 10% FCS at a concentration of  $1 \times 10^6$  cells/ml. Cells were then incubated at 37 or 4°C for the indicated times, placed on ice, washed with cold PBS containing 0.1% NaN<sub>3</sub> and 2% FCS, and stained by immunofluorescence staining for the expression of CD73, as described previously (2). (b) Lymphocytes and EC were preincubated on ice for 30 min in the presence of either 3G6, 4G4, or 1E9 mAb and washed with cold RPMI.



**Figure 1.** 1E9 and 4G4 mAbs recognize distinct epitopes of the CD73 molecule. 4G4 is a mouse IgG<sub>1</sub>, and 1E9 is a mouse IgG<sub>3</sub> mAb. (a) FACS<sup>®</sup> staining of HEC cells using 1E9 and FITC anti-mouse IgG<sub>1</sub> (anti-mouse IgG<sub>1</sub> faintly cross-reacts with IgG<sub>3</sub>, causing the low positivity). (b) FACS<sup>®</sup> staining of HEC cells using 4G4 and FITC anti-mouse IgG<sub>1</sub>. (c) HEC cells were sequentially treated with 1E9 mAb, 4G4 mAb, and FITC anti-mouse IgG<sub>1</sub>. (d) FACS<sup>®</sup> staining of HEC cells using 4G4 mAb and FITC anti-mouse IgG<sub>3</sub>. (e) FACS<sup>®</sup> staining of HEC cells using 1E9 mAb and FITC anti-mouse IgG<sub>3</sub>. (f) HEC cells were sequentially treated with 4G4 mAb, 1E9 mAb, and FITC anti-mouse IgG<sub>3</sub>. Percentage of positive cells and the mean fluorescence intensity are indicated in the upper right hand corner of each panel.

Cells were thereafter resuspended in complete medium or RPMI at 37°C and incubated for the indicated periods of time at 37°C. After this, the cells were replaced on ice, washed as described, and labeled using either both first stage mAb (4G4 or 1E9) with a second stage FITC-conjugated antibody or only a second stage FITC Ab, and analyzed by FACScan. (c) Lymphocytes were incubated in presence of 4G4 mAb or 3G6 mAb (as described in a) and analyzed by FACS<sup>®</sup>, either before or after acetone permeabilization. Cells were permeabilized by covering them for 2 min in acetone at -20°C. Cells were then pelleted, washed once with cold PBS containing 0.1% NaN<sub>3</sub> and 2% FCS and stained for the expression of CD73. (d) To study the ability of 4G4 mAb to induce patching or capping of the CD73 molecule on lymphocyte and HEC cell surface, cells were first incubated with 4G4 mAb for 20 min at 37°C and then washed twice with RPMI 1640. After this, cells were incubated at 37°C for 20 min in presence of an FITC-conjugated anti-mouse Ig diluted in RPMI 1640, washed twice with RPMI, and fixed with 1% paraformaldehyde. For comparison, lymphocytes and HEC cells were stained on ice in presence of 0.1% NaN<sub>3</sub>. Cytocentrifuge preparations of stained lymphocytes and HEC cells were analyzed under a fluorescence microscope.

### Dot-blot Assay for Detecting Cellular and Soluble CD73

Lymphocytes and EC were incubated with 4G4 mAb (as described above in b). Thereafter, either  $0.25 \times 10^6$ ,  $0.5 \times 10^6$ , and  $1 \times 10^6$  lymphocytes or  $0.3 \times 10^6$ ,  $0.6 \times 10^6$ , and  $0.9 \times 10^6$  EC were lysed in 100 µl of a buffer containing 50 mM β-octyl glucoside, 1 mM PMSF, and 1% Aprotinin (Sigma Chemical Co.) in PBS. The 100 µl aliquots of lysates were cleared by centrifugation and transferred onto a wet nitrocellulose membrane (Hybond-

ECL; Amersham Intl., Buckinghamshire, UK) using a dot blot apparatus (The Convertible, Filtration Manifold System; GIBCO BRL, Gaithersburg, MD). Antigen detection on nitrocellulose was performed as described before (25), using either mAb 4G4 at 2 µg/ml as a first stage reagent and a peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts A/S, Glostrup, Denmark) at 1:3,000 dilution (containing 5% FCS) as a second stage reagent, or only the peroxidase-conjugated rabbit anti-mouse Ig. Immunodetection was performed using an ECL detection kit for Western blotting (Amersham Intl.), following the manufacturer's instructions. Light emission was detected using Hyperfilm MP (Amersham Intl.).

### Labeling and Immunoprecipitation of Cells

Freshly isolated PBL and HECs were iodinated with <sup>125</sup>I by the lactoperoxidase method (17). Iodinated cells were lysed in lysis buffer (1% Triton X-100, 0.15 M NaCl, 1.5 mM MgCl<sub>2</sub>, and 0.01 M Tris, pH 7.2) containing 1% Aprotinin and 1 mM PMSF, and the lysate was clarified by centrifugation. Immunoprecipitation was performed using CnBr-activated Sepharose 4B beads coupled to mAb 4G4, 3G6, or Hermes-3. Antigens were eluted from the beads with 50 µl Laemmli's sample buffer and resolved in 5–12.5% SDS-PAGE under reducing conditions.

### Northern Blot Analysis, Isolation, and Sequencing of CD73 cDNA

A cDNA encoding CD73 was amplified by reverse transcription PCR (RT-PCR) from total RNA isolated from PBL. Standard techniques were used for the RNA isolation, Northern blot analyses, cDNA synthesis, PCR amplification, subcloning procedures, and plasmid preparations (29). 2 µg of PBL RNA was reverse transcribed into cDNA using random hexamer primers and MMLV reverse transcriptase. The CD73 primers 5'GGGGATCCAGTTCACGCGCCACAG3' and 5'CCCTCGAGGCAAGGAGAATTTTGG3', based on the sequence of the published placental CD73 cDNA (20), were used to amplify a full length CD73 fragment at amplification conditions of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min with 30 amplification cycles. The resulting 1.75-kb amplified fragment was blunt-end cloned into pUC18 using a SureClone kit (Pharmacia Fine Chemicals) and sequenced on both strands using gene-specific primers on an automated sequencer (Prism 377; Applied Biosystems, Foster City, CA).

### Phosphatidylinositol-specific Phospholipase C Treatment of HEC Cells

HEC cells detached with 5 mM EDTA were resuspended in RPMI 1640 in the presence or absence of *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC; Oxford Glycosystems, Oxon, UK) at 1 U/ml and incubated for 1 h under rotation at 37°C. Finally, immunofluorescence staining for CD73 expression was performed.

### Ecto-5'-NT Inhibition

Activity of ecto-5'-NT was analyzed as reported previously (3). [<sup>14</sup>C]AMP (ICN Biomedicals, Inc., Irvine, CA) was converted into <sup>14</sup>C-adenosine and <sup>14</sup>C-inosine by HEC ecto-5'-NT in presence of 30 µg/ml of purified anti-CD73 mAb 1E9 or negative control mAb 3G6. Substrate and product were separated by thin-layer chromatography, and the corresponding radioactivity was detected by autoradiography. Quantitation was performed by measuring the intensity of the dots, using the Microcomputer Imaging Device (MCID; Imaging Research Inc., Ontario, Canada).

### Detection of Tyrosine Phosphorylated Proteins after Triggering of CD73 on EC and Lymphocytes

Confluent monolayers of EC in 25 cm<sup>2</sup> cell culture flasks (~5 × 10<sup>6</sup> cells/flask) or 0.8 × 10<sup>6</sup> PBL enriched for CD73<sup>+</sup> cells (i.e., CD8<sup>+</sup>CD11b<sup>-</sup>T cells and B cells) were washed twice with PBS and then incubated for 30 min at 4°C in the presence of anti-CD73 mAb 4G4, anti-CD73 mAb 1E9, or a negative control mAb 3G6. After two washes with PBS, a rabbit anti-mouse Ig (Dako, Santa Barbara, CA), at a 20 µg/ml concentration, was added, and incubation continued for another 30 min. After washing, the cells were incubated for variable time periods at 37°C in RPMI 1640 to allow signaling, and then the cells were lysed in lysis buffer containing 10 mM EDTA and 1 mM orthovanadate (Sigma Chemical Co.; for EC, 1 ml of buffer was used and for lymphocytes, 200 µl of buffer was used).

The cell lysate was centrifuged at 13,000 rpm for 15 min at 4°C and treated with protein G-Sepharose to remove immunoglobulins. Total cellular proteins in 45 µl of lysate were separated by SDS-PAGE and transferred onto nitrocellulose. To detect tyrosine phosphorylated proteins, the filters were first incubated for 1 h in 5% BSA in TBS-T (0.05% Tween in TBS) and then immunoblotted with an anti-phosphotyrosine mAb (4G10; Upstate Biotechnology). An irrelevant mAb was used as a negative control at an equivalent concentration (0.2 µg/ml). The second-stage reagent was a biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) which was finally detected by a horseradish peroxidase biotin streptavidin conjugate (Amersham Intl.). The filters were developed using enhanced chemiluminescence.

## Results

### mAb Triggering of Lymphocyte CD73 but Not Endothelial CD73 Causes Downregulation of the Molecule at 37°C

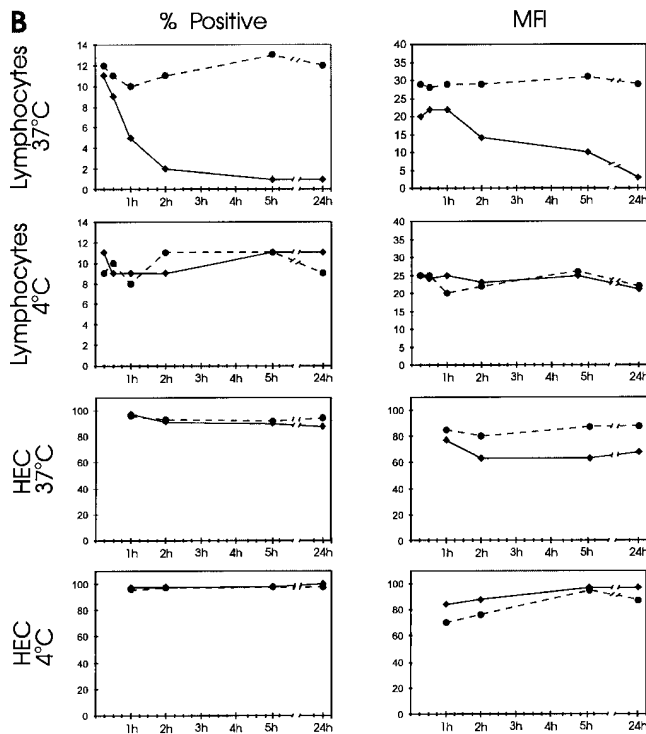
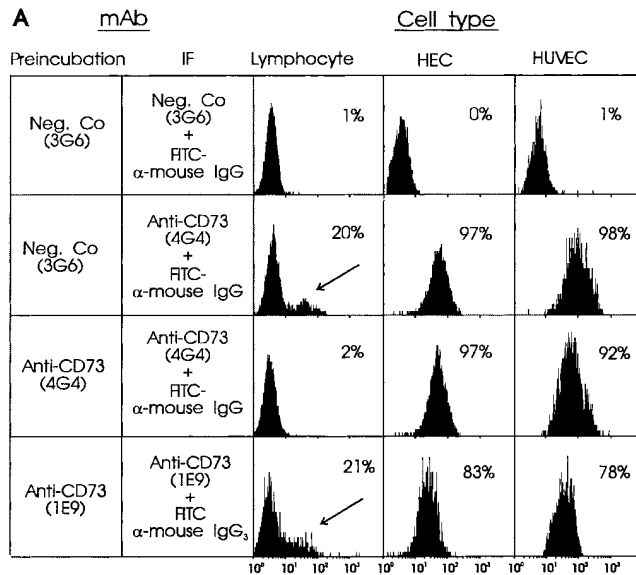
CD73 is expressed on ~15% of freshly isolated PBL and on all cultured HUVECs and HEC cells (2). To study the consequences of CD73 ligand binding in lymphocytes and EC, we used a model in which binding of CD73 to its natural ligand (which is still unknown at present) was mimicked by triggering CD73 with an mAb. In this model, lymphocytes or EC (either the HEC endothelial cell line or HUVECs, adherent or in suspension after detachment by 5 mM EDTA treatment) were incubated for 2 h at 37°C in the presence of an anti-CD73 mAb 4G4, anti-CD73 mAb 1E9, or a negative control mAb, after which CD73 expression on the cell surface was analyzed by FACS<sup>®</sup> (in the case of adherent EC, detachment was with 5 mM EDTA before immunofluorescence [IF] staining). On lymphocytes, mAb 4G4 caused a reduction in CD73 expression, but no alteration in the expression level of CD73 was observed after incubation with a negative control mAb or anti-CD73 mAb 1E9 (Fig. 2 A). On EC, no reduction in CD73 expression was seen subsequent to mAb treatments (Fig. 2 A).

### Kinetics of Surface CD73 Modulation

To study the rate of CD73 downregulation on lymphocytes, the cells were incubated in the presence of anti-CD73 mAb for different time periods and analyzed by FACS<sup>®</sup>. The downregulation of CD73 on lymphocytes was maximal after 5 h incubation (1% positive cells left), and the expression remained low during the entire 24 h follow-up period (Fig. 2 B). On EC, a minor reduction in the mean fluorescence intensity (MFI) was seen as a consequence of anti-CD73 treatment at 37°C, but the percentage of positive cells remained unaltered even after prolonged incubation (Fig. 2, A and B). Downregulation of the lymphocyte CD73 was temperature dependent, as it did not occur when the cells were incubated with the 4G4 mAb on ice (Fig. 2 B). It was also dependent on divalent cations, as no downregulation was observed when the cells were incubated with anti-CD73 mAb at 37°C without Ca<sup>2+</sup> and Mg<sup>2+</sup> (data not shown). It also is epitope dependent, as the two different anti-CD73 mAbs, 4G4 and 1E9, which recognize distinct epitopes of the molecule (Fig. 1), have profoundly dissimilar effects.

### Modulation of CD73 on EC and Lymphocyte Surface

Continuous recycling between the cell surface and the in-



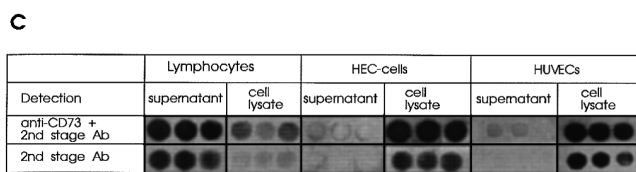
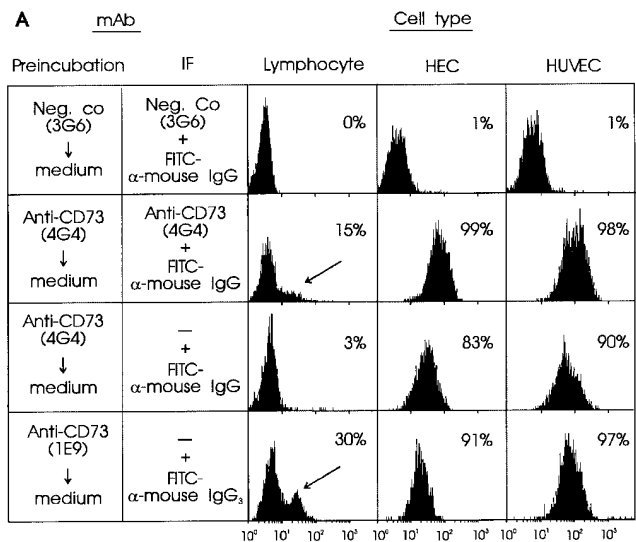
**Figure 2.** Triggering of CD73 at 37°C results in CD73 downregulation in lymphocytes but not in EC. (A) Lymphocytes, HECs, and HUVECs were incubated for 2 h in presence of an anti-CD73 mAb 4G4, anti-CD73 mAb 1E9, or a negative control mAb 3G6 at 37°C, and the expression of CD73 on the cell surface was analyzed by IF and FACS<sup>®</sup>. The arrows in the middle and bottom panels point at the CD73 positive subpopulation of PBL. (B) Downregulation of CD73 on lymphocyte surface is temperature dependent. CD73 expression was analyzed by IF staining and FACS<sup>®</sup> after incubating lymphocytes and HECs in presence of an anti-CD73 (◆, continuous line) or in presence of a negative control mAb (●, dashed line) for various time periods at 37° or 4°C. Percentage of positive cells is shown in the graphs on the left, and the MFI of positive cells is shown in the graphs on the right.

tracellular pool has been described for ecto-5'-NT in rat fibroblasts and hepatocytes (31, 41, 45). We studied whether similar spontaneous recycling of CD73 would take place in EC. Triggering of CD73 with an mAb does not seem to greatly affect the level of surface expression of the molecule on EC, as shown in Fig. 2. However, when EC, which had been surface labeled with anti-CD73 mAb on ice, were incubated at 37°C in fresh medium, it was seen that a considerable portion of the CD73 molecules was removed from the cell surface, and new CD73 molecules appeared on the surface as a replacement. This was seen as a markedly lower net MFI after detection with only an FITC anti-mouse Ig as compared to the net MFI after detection with both anti-CD73 mAb and a second stage Ab (Fig. 3 A). The percentage of positive cells was only slightly reduced, suggesting that only a portion of CD73 molecules on each individual cell is removed from the surface, while the rest are unaffected (Fig. 3 A). Thus, in the case of the EC, binding of CD73 does not seem to affect its surface expression, but rather, the results suggest that the CD73 molecules are recycling.

When lymphocytes were treated with 4G4 mAb at a saturating concentration at 4°C, washed and then incubated in complete medium without anti-CD73 mAb at 37°C for 15 min, a rapid loss of the antibody-bound CD73 molecules from the cell surface and appearance of new, non-Ab-bound CD73 molecules was seen. This was shown as a smaller percentage of fluorescent cells when lymphocytes, after incubation at 37°C, were labeled only with an FITC-conjugated anti-mouse Ig and analyzed by FACS<sup>®</sup>, as compared to the percentage of positive cells when the cells were labeled with both an anti-CD73 mAb and an FITC-anti-mouse Ig (Fig. 3 A). The loss of antibody-bound CD73 from lymphocyte surfaces was very rapid, as the percentage of positive cells was already reduced after a 2 min incubation in complete medium (data not shown). Kinetic studies show that after incubating lymphocytes for up to 5 h in complete medium, the level of antibody-bound, surface CD73 was still reduced (i.e., the antibody-bound molecules do not return to the cell surface once they are removed, Fig. 3 B). Binding of anti-CD73 mAb 1E9, again, causes no reduction in the CD73 expression on lymphocytes (Fig. 3 A).

#### mAb Binding to Lymphocyte CD73 but Not to Endothelial CD73 Causes Shedding of the Molecule

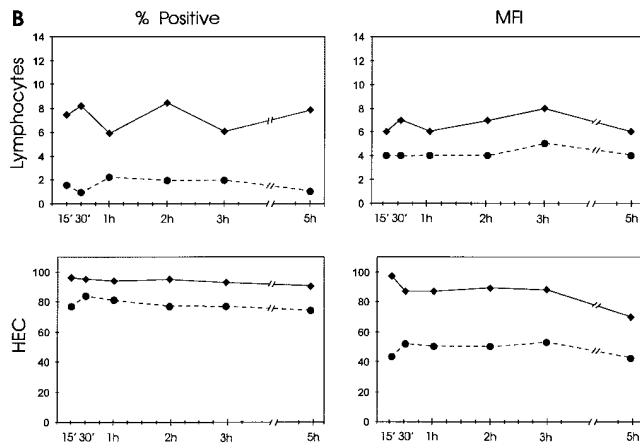
To determine whether CD73 on lymphocytes and EC is shed or internalized, dot blot assays were performed. Lymphocytes and EC were incubated on ice in the presence of anti-CD73 mAb 4G4, washed twice, and then incubated in RPMI at 37°C for 1 h. After the incubation, CD73 was detected either from the supernatant or from the cell lysate using 4G4 mAb as a first stage mAb and peroxidase-conjugated anti-mouse Ig as a second stage mAb, or by performing the detection using only the second stage Ab. As can be seen in Fig. 3 C, CD73 could be detected in the lymphocyte supernatant using only the second stage Ab in the dot blot assay, indicating that the molecule initially present on the cell surface had been shed into the supernatant after mAb binding. Signals from lysed lymphocytes are weaker, suggesting that only low amounts of



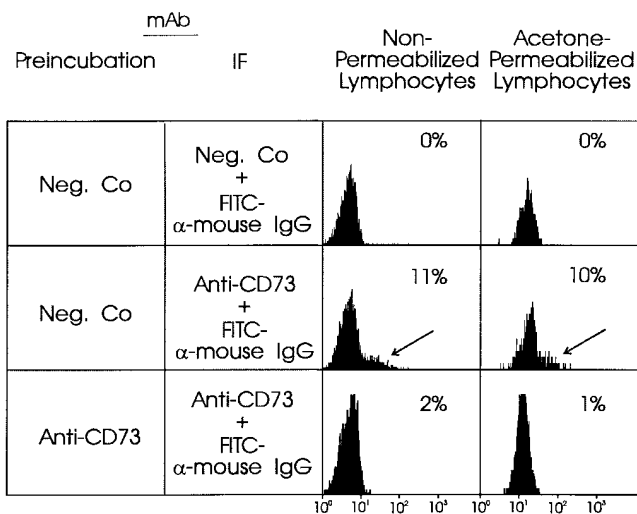
time. After incubation, cells were either labeled with both anti-CD73 mAb and a second stage, FITC-conjugated Ab (◆, *continuous line*) or only with a second stage, FITC-conjugated Ab (●, *dashed line*). MFI is measured for all cells. (C) CD73 can be detected from the lymphocyte supernatant but not from the EC supernatant. Lymphocytes, HECs, and HUVECs were incubated with the anti-CD73 mAb as described above, and the cell lysate and the incubation supernatant were analyzed for presence of CD73 using either both a primary and a secondary Ab or only a secondary Ab in a dot blot assay, as described in Materials and Methods. The three spots in each panel represent different numbers of cells. For lymphocytes: left spot,  $1 \times 10^6$  cells, middle spot,  $0.5 \times 10^6$  cells, and right spot,  $0.25 \times 10^6$  cells except that the right spot of the cell lysate detected with both first and second stage Abs has  $1.5 \times 10^6$  cells. For EC: left spot,  $0.9 \times 10^6$  cells, middle spot,  $0.6 \times 10^6$  cells, and right spot,  $0.3 \times 10^6$  cells.

CD73 are left in the cell after mAb treatment at 37°C. On the other hand, no CD73 could be detected from the EC supernatant, but the CD73 signals from the EC lysate were strong (Fig. 3 C). Thus, CD73 on EC is not removed from the cell surface due to mAb binding, and EC do not spontaneously secrete CD73. Instead, the reduction of mAb-bound CD73 on EC surface after resuspending the cells in medium at 37°C, is probably due to internalization of the molecule, as in the dot blot experiment in which signals are much stronger when detection is performed using both first stage and second stage mAb as compared to detection only with the second stage mAb (Fig. 3 C). To further confirm that continuous exposure of lymphocytes to anti-CD73 Ab at 37°C causes shedding instead of internalization of CD73, lymphocytes were treated with mAb, permeabilized with acetone, and then stained with anti-CD73 mAb to study whether intracellular CD73 could be detected. As can be seen in Fig. 4, after anti-CD73 preincubation at 37°C, no CD73 could be detected on either nonpermeabilized or acetone-permeabilized lymphocytes. If the cells were incubated in the presence of a negative control mAb, CD73 was present both on the non-permeabilized cells and in the acetone-permeabilized cells.

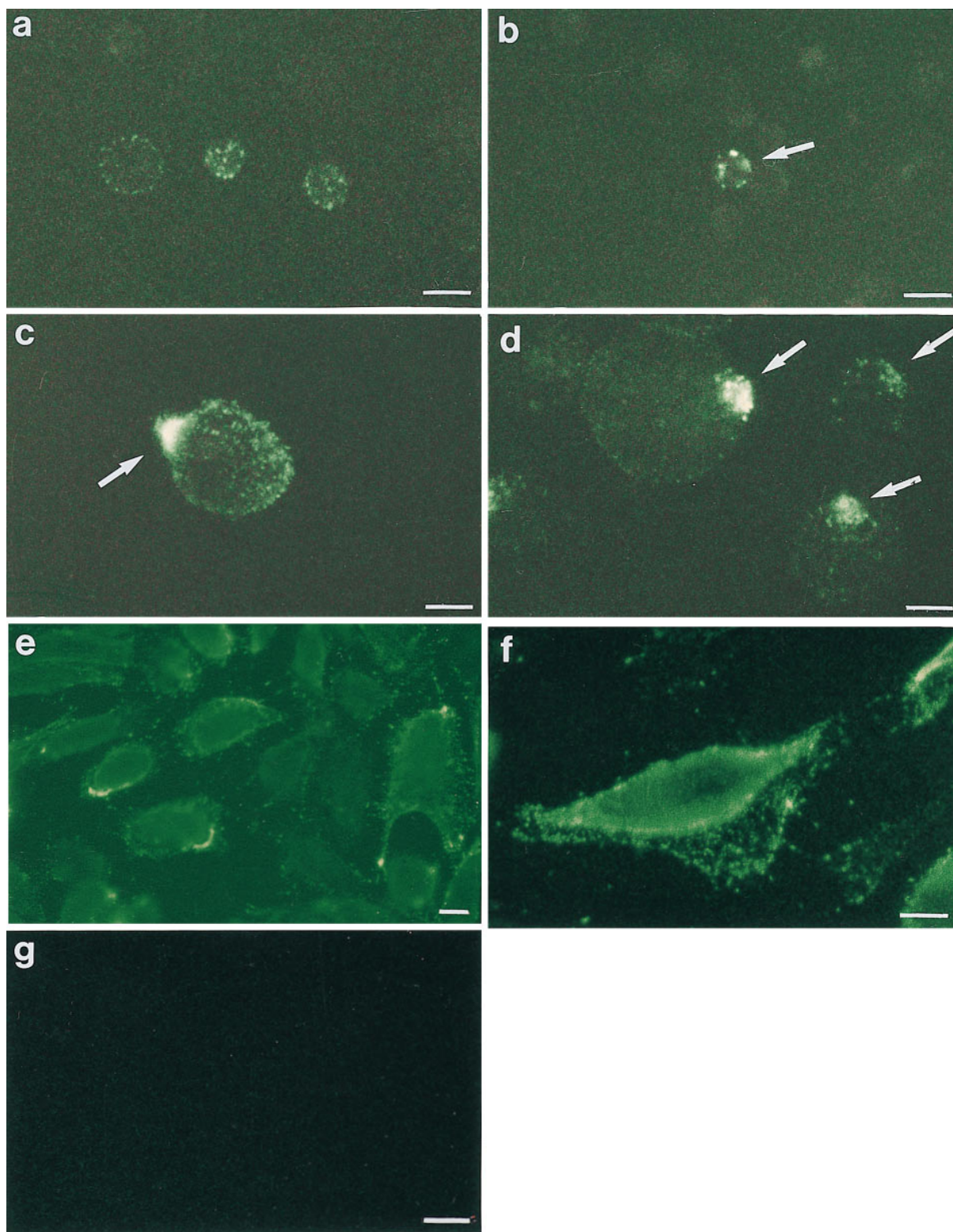
We thus conclude that CD73 molecules are shed from the lymphocyte surface upon antibody binding, and new CD73 molecules from inside the cell replace those which



**Figure 3.** Downregulated CD73 is rapidly replaced by new CD73 molecules. (A) Lymphocytes, HECs, and HUVECs in suspension were preincubated with negative control mAb, anti-CD73 mAb 4G4, or anti-CD73 mAb 1E9, at 4°C; washed twice and incubated in complete medium at 37°C for 15 min; and then stained with either anti-CD73 mAb and a second stage FITC-conjugated Ab, or only with a second stage Ab. The arrows in the middle and bottom panels point at the CD73 positive subpopulation of PBL. (B) FACScan® analysis of lymphocytes and HECs after a saturating preincubation on ice with an anti-CD73 mAb and a subsequent incubation in complete medium at 37°C for various periods of



**Figure 4.** CD73 is not detectable in permeabilized lymphocytes after mAb 4G4 treatment at 37°C. FACS® analysis was performed with permeabilized and non-permeabilized lymphocytes after 2 h incubation at 37°C in presence of either anti-CD73 mAb 4G4 or negative control mAb. The arrows in the middle panel point at the CD73-positive subpopulation of PBL.



**Figure 5.** Cross-linking of CD73 results in different staining patterns on lymphocyte and endothelial surfaces. Lymphocytes and EC were stained with mAb 4G4 and a second stage FITC-conjugated Ab, as described in Materials and Methods. (a) Patching of CD73 on lymphocyte surface after cross-linking at 4°C. (b) At 37°C, the majority of lymphocytes becomes CD73 negative due to CD73 cross-linking, and the remaining CD73-reactivity is found in large patches (arrow). (c) Capping of CD73 on EC after cross-linking at 4°C (arrow). (d) Capping of CD73 on EC after cross-linking at 37°C (arrows). (e) Punctate staining pattern of adherent HEC cells stained with anti-CD73 mAb at 4°C. (f) Punctate staining pattern of adherent HEC cells stained with anti-CD73 mAb at 37°C. (g) Adherent HEC cells stained with a negative control mAb 3G6 at 37°C. In a, b, c and d, cells were stained in suspension and analyzed microscopically on cytospin preparations. Bar, 10 μm.

are lost. When extra Ab is available at 37°C to bind newly surface-located molecules, the internal pools of CD73 molecule are depleted (or the synthesis of CD73 is down-regulated). Thus, no molecules are available to replace those which are shed, and the cells become completely CD73 negative.

### Cross-linking of CD73 on Lymphocytes Causes Patching, Whereas Cross-linking of CD73 on EC Causes Capping

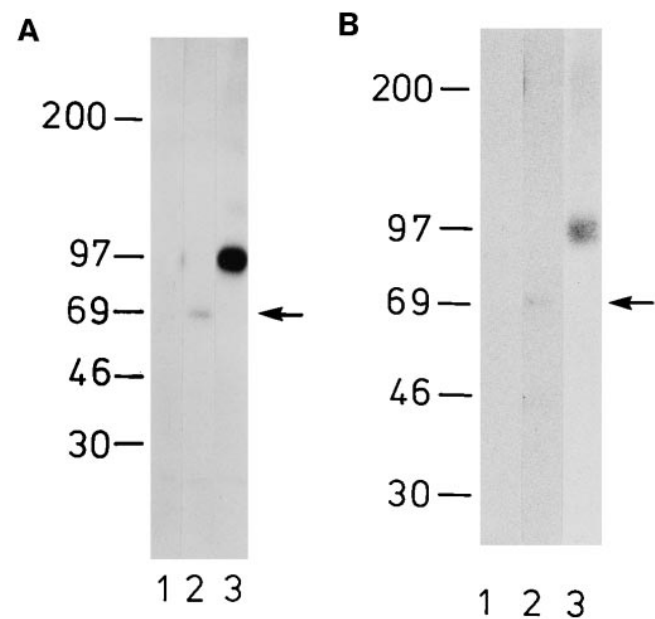
When PBL were incubated sequentially with an anti-CD73 mAb and a cross-linking FITC-conjugated second stage Ab, at 4°C, patching of CD73 was observed on the lymphocyte surface when cells were analyzed by fluorescent microscopy (Fig. 5 A). When the same procedure was performed at 37°C, a substantial number of the cells became CD73 negative, and the remaining CD73 reactivity was found in large clusters (Fig. 5 B). On EC, partial capping of CD73 can be seen after antibody cross-linking at 4°C, but substantial reactivity can still be found all around the cell (Fig. 5 C). After cross-linking at 37°C, the capping of CD73 to one pole is complete, and practically no CD73 reactivity can be detected on other parts of the cell (Fig. 5 D). Adherent HEC cells display a granular staining pattern after CD73 cross-linking both at 4°C (Fig. 5 E) and at 37°C (Fig. 5 F).

### Immunoprecipitation of CD73 from Lymphocytes and EC

We have shown above, by several complementary methods, how CD73 surface expression is differentially modulated following Ab binding and incubation at 37°C in lymphocytes and EC. To elucidate whether structural divergence between lymphocyte and endothelial CD73 could explain the differences, we immunoprecipitated the CD73 molecule from <sup>125</sup>I-labeled lymphocytes and EC. However, no significant differences in the size of the CD73 molecule were observed (Fig. 6). The protein migrates as a single, 70-kD band in 5–12.5% SDS-PAGE gels under reducing conditions, whether it is derived from EC or PBL.

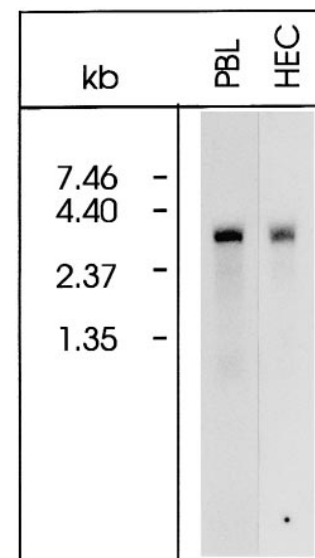
### Northern Blot Analyses, Cloning, and Sequencing of Lymphocyte and Endothelial CD73 cDNA

Although no gross differences at the protein level were detected in CD73 expressed on different cell types, we examined polyA<sup>+</sup> RNA isolated from both lymphocytes and HEC cells by Northern blot analysis to determine if there is any size divergence at the mRNA level. In both HEC and PBL mRNAs, a strong band of ~3.9 kb was detected (Fig. 7). Despite similar amounts of polyA<sup>+</sup> RNA (10 µg/lane), the signal detected from lymphocyte RNA was severalfold stronger, possibly indicating a much higher turnover rate of lymphocyte CD73 as compared to the EC CD73. Prolonged exposure of the blot revealed the presence of smaller, hybridizing bands in both samples which may represent minor CD73 transcripts, prematurely truncated mRNAs, or degradation products. To compare the sequences of CD73 HEC and PBL cDNAs, we isolated a full length CD73 cDNA from lymphocytes by PCR and completely sequenced both this and the previously cloned

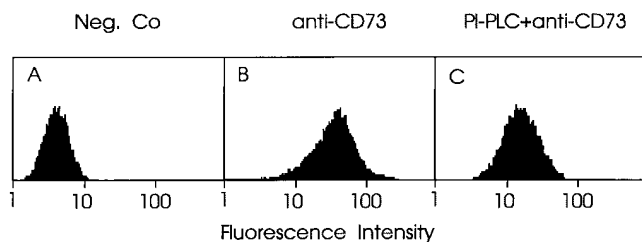


**Figure 6.** Molecular mass of CD73 from lymphocytes and EC. (A) <sup>125</sup>I-labeled HECs were lysed, and immunoprecipitations were performed with a negative control mAb (lane 1), mAb 4G4 (lane 2, arrow), and mAb Hermes-3, which recognizes the CD44 molecule (lane 3) as described in Materials and Methods. (B) Lysoates from freshly isolated <sup>125</sup>I-labeled PBL were immunoprecipitated with a negative control mAb (lane 1), mAb 4G4 (lane 2, arrow), and mAb Hermes-3. No significant size differences were observed between CD73 molecules from lymphocytes and EC.

endothelial form from HEC cells (2). No significant differences were found in the nucleotide sequences, which were almost identical to the previously published human CD73 cDNA sequence from placental tissue (21). A single nucleotide change, A > G<sup>1175</sup>, was found in both HEC and PBL cDNAs, resulting in a threonine to alanine change, and an



**Figure 7.** Northern blot analysis of lymphocyte and endothelial CD73 mRNA. PolyA<sup>+</sup> RNA (10 µg/lane), prepared from human PBL and from cultured HECs, was denatured and separated by electrophoresis on a 1.1% agarose gel. The RNA was blotted onto a Hybond N membrane and hybridized with a <sup>32</sup>P-labeled CD73 cDNA probe. Posthybridization wash conditions were 0.1× SSC, 0.1% SDS at 65°C for 1 h. SSC is 0.15 M NaCl, 0.015 M Na citrate. The signal detected from lymphocyte RNA was several times stronger than that from HEC RNA, and thus, the exposure time for the PBL blot is 12 h and the exposure time for the HEC blot is two weeks.



**Figure 8.** PI-PLC treatment of HEC cells. HEC cells in suspension were treated with PI-PLC and analyzed by immunofluorescence staining and FACS<sup>®</sup> for the expression of CD73. Negative control staining was performed using a class-matched, nonbinding mAb as the first stage antibody.

A > C<sup>730</sup> in HEC cDNA, resulting in a lysine to asparagine substitution. These may most likely be attributed to PCR amplification errors or polymorphism due to the different tissue materials used.

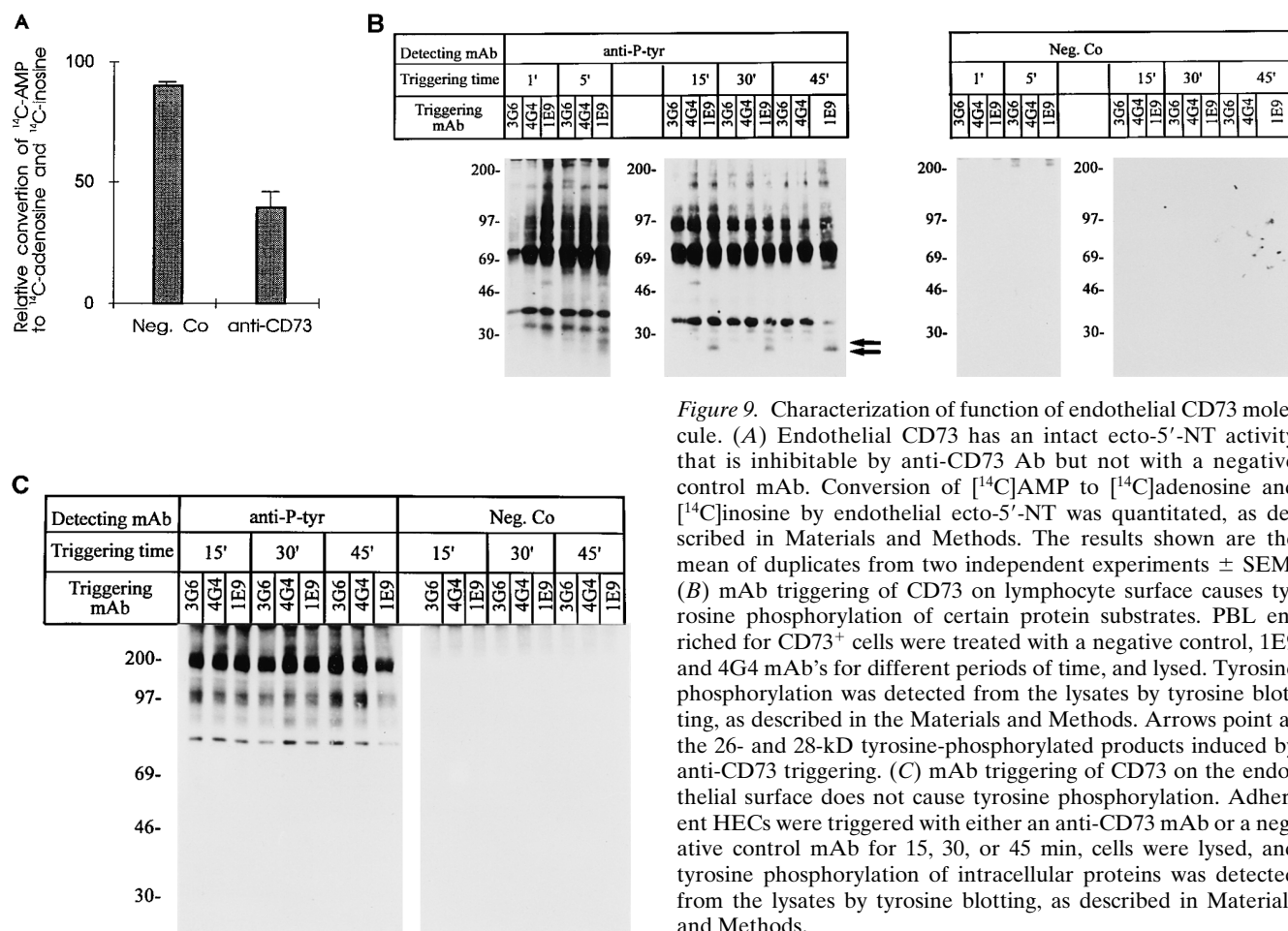
### PI-PLC Treatment of HEC Cells

We next addressed the question of whether differences in the anchoring of CD73 to the plasma membrane of the cell could be observed between lymphocytes and EC. CD73 is known to be a GPI-linked molecule, and it has been previously shown that lymphocyte CD73 can be cleaved off by

PI-PLC treatment of cells (3, 40). To study the susceptibility of CD73 to PI-PLC in EC, HEC cells were treated with PI-PLC, immunofluorescence stained with anti-CD73 mAb, and analyzed by FACS<sup>®</sup> (Fig. 8). A significant proportion of CD73 on HEC cells was resistant to PI-PLC as the MFI was only slightly decreased by enzyme treatment. It is probable that each individual cell has both PI-PLC-susceptible and PI-PLC-resistant CD73 molecules on its surface as, although the MFI of the cells was reduced by PI-PLC-treatment, no significant changes were seen in the number of positive cells. Internalization of CD73 on HEC cells at 37°C does not seem to be dependent on the PI-PLC susceptibility of the molecule because the internalization could also be observed after the PI-PLC treatment (data not shown).

### Inhibition of EC Ecto-5'-NT Activity by an Anti-CD73 mAb

To demonstrate the nucleotidase activity of CD73 expressed on EC, i.e., the ability of CD73 to hydrolyze extracellular mononucleotides to their corresponding nucleosides, we performed enzyme assays by adding [<sup>14</sup>C]-labeled AMP as a substrate to HEC cell cultures and then detecting [<sup>14</sup>C]adenosine and [<sup>14</sup>C]inosine as products of the enzyme activity. High amounts of [<sup>14</sup>C]adenosine and [<sup>14</sup>C]inosine were generated in the presence of a negative



**Figure 9.** Characterization of function of endothelial CD73 molecule. (A) Endothelial CD73 has an intact ecto-5'-NT activity that is inhibitable by anti-CD73 Ab but not with a negative control mAb. Conversion of [<sup>14</sup>C]AMP to [<sup>14</sup>C]adenosine and [<sup>14</sup>C]inosine by endothelial ecto-5'-NT was quantitated, as described in Materials and Methods. The results shown are the mean of duplicates from two independent experiments ± SEM. (B) mAb triggering of CD73 on lymphocyte surface causes tyrosine phosphorylation of certain protein substrates. PBL enriched for CD73<sup>+</sup> cells were treated with a negative control, 1E9 and 4G4 mAb's for different periods of time, and lysed. Tyrosine phosphorylation was detected from the lysates by tyrosine blotting, as described in the Materials and Methods. Arrows point at the 26- and 28-kD tyrosine-phosphorylated products induced by anti-CD73 triggering. (C) mAb triggering of CD73 on the endothelial surface does not cause tyrosine phosphorylation. Adherent HECs were triggered with either an anti-CD73 mAb or a negative control mAb for 15, 30, or 45 min, cells were lysed, and tyrosine phosphorylation of intracellular proteins was detected from the lysates by tyrosine blotting, as described in Materials and Methods.



control antibody, whereas when an anti-CD73 workshop Ab 1E9 was present, the nucleoside production was inhibited by 60% (Fig. 9 A). This demonstrates that CD73 on EC has high ecto-5'-NT activity. Ecto-5'-NT activity of CD73 expressed on human PBL has been shown earlier (3, 40).

### **Antibody Triggering of CD73 Induces Tyrosine Phosphorylation of Certain Proteins in Lymphocytes but Not in EC**

Tyrosine phosphorylation and dephosphorylation of several proteins has been demonstrated due to mAb triggering of lymphocyte CD73 (9). To compare the signal transducing abilities of CD73 in lymphocytes versus EC, we triggered CD73 on both cell types with mAb's, and analyzed the alterations in patterns of tyrosine phosphorylated proteins by western blotting total cellular lysates. For lymphocyte triggering we used a population of freshly isolated, CD8<sup>+</sup>CD11b<sup>-</sup> lymphocytes, among which CD73<sup>+</sup> cells are highly enriched; and thus, the detection of CD73-mediated signals is facilitated. In lymphocytes the appearance of an ~26-kD phosphorylated substrate was observed 5 min after beginning triggering with anti-CD73 mAb 1E9 (time point zero is when the unbound antibody is washed away and the cells are resuspended in warm medium to allow signaling). The phosphorylation peaked at 15 min and was still clearly visible at 45 min. At the 5 min time point, 1E9 also triggered stronger phosphorylation of a 28-kD protein, as compared to negative control mAb. The phosphorylation of the 28-kD protein was strongest at 30 min and weaker at the longest triggering time of 45 min. Induction of a tyrosine phosphorylated protein of the same size and with similar kinetics has also been reported earlier due to anti-CD73 triggering (9). The appearance of these 26- and 28-kD bands was specifically due to triggering with 1E9 mAb since no such induction was observed after triggering with an irrelevant control antibody 3G6 or mAb 4G4 under the same conditions (Fig. 9 B). In addition, induction of an ~50-kD phosphorylated protein was seen at shorter time points (1 and 5 min). A 50-kD phosphorylated band was also visible 15 min after commencing the triggering with 4G4 mAb. When EC were triggered under similar conditions, no differences in the phosphotyrosine blots were seen when the triggering was performed using either an irrelevant control Ab or an anti-CD73 mAb 1E9 for 15, 30, or 45 min (Fig. 9 C). Triggering of EC with an anti-CD73 or a negative control mAb was also performed for shorter periods of time (data not shown), but this also had no effect. This suggests that the CD73 molecule has different signal transducing properties in lymphocytes and EC. In lymphocytes, a phosphotyrosine pathway is involved, but whether CD73-mediated signaling plays any role in EC remains to be seen.

### **Discussion**

To elucidate the properties of CD73 in lymphocytes and EC, the key effector cells in lymphocyte homing and in many other important immunological phenomena, we have studied the endothelial and lymphocyte CD73 molecules in terms of (a) expression and its modulation; (b) structure

at the protein, mRNA, and cDNA levels; and (c) signaling and enzymatic functions. The differences we observed between lymphocyte and endothelial CD73 imply that it has profoundly dissimilar functional properties in these different cell types.

As the natural ligand of CD73 is not known, mAb binding to CD73 was used to mimic ligand engagement in this study. It was shown that CD73 is downregulated from the lymphocyte surface due to mAb 4G4 binding at 37°C (see Fig. 2). When lymphocytes are cultured in fresh medium at 37°C, after being labeled at 4°C with anti-CD73 mAb, the antibody-bound CD73 molecules are shed from the cell surface into the supernatant, as CD73 can be detected in the supernatant using only a secondary Ab in the dot blot assay. The shed molecules are rapidly replaced by new CD73 molecules, which probably come from intracellular stores, or are newly synthesized (see Fig. 3). If there is new anti-CD73 Ab available, the shedding continues and the cells become completely depleted of both surface and intracellular CD73. Partial downregulation (both endocytosis and shedding) of other GPI-linked molecules, like decay accelerating factor, Thy-1, and Ly6A from the leukocyte surface due to mAb binding at 37°C, has been described earlier (5, 34). No alteration was seen in the expression of lymphocyte CD31 after similar antibody triggering (data not shown), indicating that surface modulation as a consequence of ligand binding is a specific property of only certain molecules. In the case of the GPI-linked molecules, either endogenous phospholipase C or vesiculation might be responsible for the release of the molecule from the plasma membrane. In sharp contrast to lymphocytes, no downregulation of CD73 due to mAb binding at 37°C was observed in EC. However, if EC were labeled with anti-CD73 mAb at 4°C, and subsequently resuspended in fresh, warm medium, a reduction in the fluorescence intensity was observed after staining the cells with only a secondary Ab rather than with both an anti-CD73 mAb and a secondary Ab. This indicates that some of the CD73 molecules initially present on the endothelial surface are removed after placing the cells in warm medium, and that these molecules are replaced by new molecules coming from intracellular stores. The reduction of CD73 on EC, however, is not caused by mAb binding but is probably an indication of spontaneous recycling of the molecule between intracellular pools and the cell surface, as has been described for ecto-5'-NT in rat fibroblasts, hepatocytes, and a hepatoma cell line (31, 41, 45).

No major differences were observed at the protein level when lymphocyte and endothelial CD73 molecules were compared, i.e., both molecules have a molecular mass of ~70 kD. Previously reported immunoprecipitations of CD73 have been performed from lysates of lymphocytic cell lines expressing CD73, and the molecular masses reported have been comparable to that now detected on lymphocytes (10, 13, 40). Northern blot analyses on HEC and PBL polyA<sup>+</sup> RNA were also performed to detect the CD73 mRNA in both cell types. The size of the mRNA detected was 3.9–4.0 kb, which corresponds well to the reported 4.1-kb size of CD73 mRNA from human placenta (21). At the nucleotide sequence level, the cDNAs originating from lymphocytes and EC were practically identical to each other and to the previously published placental form.

Several GPI-linked molecules, including CD73, have been shown to be capable of transmitting activation signals to leukocytes (27). The mechanisms of signal transduction mediated by these molecules lacking intracellular domains have been difficult to understand. But recent studies have shown that GPI-linked molecules can be expressed on the cell surface in clusters closely associated with other molecules, which often include tyrosine kinases of the src family (12, 19, 32). Thus, interaction with the src family protein kinases is one possible means for the GPI-anchored molecules to participate in the signal transduction system of the cell (22, 35). We have now shown that CD73 on EC was unable to deliver a tyrosine phosphorylation inducing signal upon mAb triggering, whereas triggering of lymphocyte CD73 induced tyrosine phosphorylation (see Fig. 9; 9). It is possible that the association of CD73 in clusters with other signaling molecules is strong in lymphocytes and less frequent in EC, and this is responsible for the different signal transducing abilities. The different clustering/capping tendencies of CD73 on lymphocytes versus EC, demonstrated by fluorescence microscopy (Fig. 5), also support the view that the subcellular localization and association with other cellular molecules is critical for the physiological responses mediated by the molecule.

As CD73 has been shown to be involved in controlling lymphocyte binding, it is possible that concentrating several CD73 molecules together in large clusters facilitates the adhesion mediated by this molecule. Alternatively, CD73 on lymphocytes could function as a co-regulatory molecule, in which case signals mediated by ligand binding to CD73 might lead to activation of other adhesion molecules. Interestingly, co-localization of  $\beta$ 2-integrins in GPI-linked molecule rich clusters has been described, and thus, activation of LFA-1 may be controlled by interaction with some of the cluster-associated molecules (7). Accordingly,  $\beta$ 1-integrin-mediated cell adhesion was recently shown to be regulated by the urokinase receptor, a GPI-linked cell surface protein (44). We have previously suggested a connection between LFA-1 and CD73, because no additive inhibition in lymphocyte binding to EC was seen when both anti-LFA-1 and 4G4 mAbs were present in the adhesion assay, as compared to either of them alone (2). It is thus possible that CD73 signalling plays a role in the regulation of LFA-1 activity and in this way, has importance in regulating lymphocyte emigration. This is supported by our recent results, which show that triggering of lymphocytes with anti-CD73 mAb 4G4 increases lymphocyte adhesion to cultured EC by  $\sim$ 50% in an LFA-1-inhibitable manner, suggesting that CD73 has an important role in regulating LFA-1-mediated lymphocyte binding (Airas, L., J. Niemelä, and S. Jalkanen, manuscript in preparation). Shedding of CD73 from the lymphocyte surface is likely to have profound importance for the regulation of LFA-1 activity, as triggering with the anti-CD73 mAb 1E9, which does not cause shedding of CD73, also fails to have the LFA-1-activating effect. Furthermore, we have recently shown that CD73 on lymphocytes plays an important role in mediating binding of lymphocytes to venules in inflamed skin, whereas CD73 on EC has minimal importance in this (4).

Several adhesion molecules are known to have alternative forms which are GPI linked to the cell surface, but not much is known about the functional importance of these

modifications. We believe that GPI-linked molecules, being very mobile, can provide an efficient way of controlling the migratory process of lymphocytes, and we suggest that the rapid shedding of lymphocyte CD73 due to ligand binding represents a novel mechanism for regulating lymphocyte-EC contacts in the high endothelial venule. The fact that the two anti-CD73 mAbs which recognize different epitopes of the molecule (i.e., 4G4 and 1E9), have different effects on the shedding of CD73, also suggests that the physiological functions mediated through CD73 are dependent on the binding of the ligand(s) to distinct epitopes of CD73. Ligand-induced shedding, mimicked by mAb binding, has also been previously described for the CD44 molecule both in vitro and in vivo, and, interestingly, only certain anti-CD44 mAb's are able to induce the release of the molecule. In vivo, the shedding of CD44 has been suggested to have importance in controlling extravasation of lymphocytes at inflammatory sites (6). Furthermore, L-selectin shedding is a well known phenomenon, and, recently, inhibition of the shedding was shown to impair neutrophil rolling (43).

Indicative of the data presented, CD73 in EC might have a completely different function from CD73 in PBL. Electron microscopy studies have revealed ecto-5'-NT activity both at the basal and the luminal side of EC in intact venules (26). As ecto-5'-NT has been shown to bind to laminin and fibronectin, it is possible that ecto-5'-NT facilitates the attachment of EC to the basement membrane (33). The ecto-5'-NT enzyme activity in EC is also quite high (Fig. 9 A), and thus the ecto-enzyme function in the vessels probably also has physiological significance.

In conclusion, these studies support the view that structurally identical CD73 molecules expressed on different cells and tissues have different functions. The enzymatic function of CD73 (or ecto-5'-NT) is to hydrolyze extracellular nucleotides to their corresponding nucleosides, but there is an increasing amount of evidence for other functions for the CD73 molecule (46). In particular, a role in mediating adhesion of different cell types and a co-signaling role in T cell activation and proliferation are of interest. Importantly, the suggested functions of CD73 do not mutually exclude each other; instead, they give a clear example of how the physiological role of a molecule can vary between different cells and organs. The differential modulation of CD73 in various cell types likely has fundamental importance in the regulation of these divergent functions.

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