A Role for Phosphatidylinositol 3-Kinase in the Regulation of β1 Integrin Activity by the CD2 Antigen

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Abstract. The rapid and reversible upregulation of the functional activity of integrin receptors on T lymphocytes is a vital step in the adhesive interactions that occur during successful T cell recognition of foreign antigen and transendothelial migration. Although the ligation of several different cell surface receptors, including the antigen-specific CD3/T cell receptor complex, the CD2, CD7, and CD28 antigens, as well as several chemokine receptors, has been shown to rapidly upregulate integrin function, the intracellular signaling events that initiate this increase in adhesion remain poorly defined. In this study, we have used DNA-mediated gene transfer to explore the role of phosphatidylinositol 3-kinase (PI 3-K) in the upregulation of β1 integrin functional activity mediated by the CD2 antigen. CD2 was expressed in the myelomonocytic cell line HL60, which expresses β1 integrins that mediate adhesion to fibronectin and VCAM-1 in an activation-dependent manner. Antibody stimulation of CD2 expressed on HL60 transfectants resulted within minutes in increased β1-mediated adhesion to fibronectin and VCAM-1 at levels comparable to that obtained upon stimulation with the phorbol ester PMA. A role for PI 3-K in CD2-mediated increases in β1 integrin function is suggested by: (a) the ability of the PI 3-K inhibitor wortmannin to completely inhibit CD2-induced increases in β1 integrin activity; (b) the association of PI 3-K with CD2; and (c) induced PI 3-K activity upon CD2 stimulation. The mode of association of PI 3-K with CD2 is not mediated by tyrosine phosphorylation-dependent binding of PI 3-K via SH2 domains, since: (a) PI 3-K is associated with CD2 in unstimulated cells; (b) CD2 stimulation fails to increase the amount of associated PI 3-K; and (c) the CD2 cytoplasmic domain lacks tyrosine residues. A role for both protein kinase C and cytoskeletal rearrangements in CD2 regulation of integrin activity is also suggested, since a PKC inhibitor partially inhibits CD2-induced increases in β1 integrin function, and CD2 stimulation increases F-actin content in a wortmannin-sensitive manner. Analysis of human peripheral T cells indicated that CD2 stimulation also results in PI 3-K–dependent upregulation of β1 integrin activity. Thus, these results demonstrate that CD2 can function as an adhesion regulator in the absence of expression of the CD3/T cell receptor complex; and directly implicate PI 3-K as a critical intracellular mediator involved in the regulation of β1 integrin functional activity by the CD2 antigen.

The precise regulation of the interaction of immune cells, such as T lymphocytes, with other cells and with components of the extracellular matrix (ECM) is vital to the successful response of the immune system to a foreign challenge. Integrin adhesion receptors are a major group of adhesion molecules used by circulating leukocytes to interact with and respond to the extracellular environment (32, 67). Since the primary function of a T cell involves recirculation in an ongoing search for foreign antigen, it is not surprising that T cells use several mechanisms of regulating integrin receptor functional activity (41). One major mode of regulating integrin function that is used by T cells as well as other circulating leukocytes involves a rapid increase in integrin functional activity after activation of the cell (17, 63, 78). Thus, while human T cells bind poorly to most integrin ligands and counter-receptors, such as the LFA-1 counter-receptor ICAM-1, the αβ1 counter-receptor VCAM-1, and the αβ1 and αβ1 ECM ligand fibronectin (FN), activation quickly increases integrin-mediated T cell function (17, 60, 62, 63, 70, 78, 82). Such activation-dependent upregulation of in-
The integrin function has been proposed to be a key step in: (a) recognition of foreign antigen by T cells, by serving to rapidly strengthen the interaction between T cells and antigen-presenting cells (17, 62); and (b) T cell recirculation and migration into inflammatory sites. Recent multi-step models of leukocyte interactions with endothelium propose that integrin upregulation represents the mechanism by which stable shear-resistant adhesion of leukocytes with endothelium is initiated and maintained (68). Consequently, further analysis of the mechanisms by which activation regulates integrin activity is essential to our overall understanding of the immune response.

Activation-dependent upregulation of integrin activity is characterized by several features. First, activation-induced increases in integrin activity occur rapidly, typically within seconds to minutes after activation (17, 63, 78). Second, activation induces changes in integrin functional activity, as measured by adhesion to relevant ligands or counter-receptors, without a change in the level of cell surface integrin expression (17, 63, 78). This suggests some qualitative alteration in the integrin receptor itself, such as a conformational change that alters the affinity of an integrin for a ligand, and/or cellular responses, such as receptor microclustering, that alter the avidity of integrin-dependent cellular interactions (18, 37). Third, multiple different receptors on a lymphocyte are capable of delivering a signal that upregulates integrin activity. We define such molecules as “integrin regulators.” On T cells, the list of known integrin regulators is now quite extensive and minimally includes the antigen-specific CD3/T cell receptor complex, CD2, CD7, CD28, CD31, as well as receptors for chemokines, such as MIP-1β and hepatocyte growth factor (1, 17, 61, 63, 71, 72, 78). Despite the growing list of known integrin regulators on T cells, the intracellular signals generated upon engagement of these molecules that lead to integrin upregulation remain poorly defined. The ability of pharmacologic agents such as the phorbol ester PMA and Ca^{2+} ionophore to upregulate integrin activity implicates both protein kinase C (PKC) and intracellular calcium with this regulatory event (17, 61, 63, 82). Studies with signal transduction inhibitors have implicated tyrosine kinases, protein kinase A, and G proteins in activation-dependent integrin upregulation (4, 12, 17, 61). However, it is not clear whether all of the integrin regulators expressed on a given cell use common or distinct signaling pathways to mediate integrin upregulation.

The present study focuses on an analysis of the intracellular signals generated by the CD2 antigen that lead to upregulation of β1 integrin activity. The CD2 antigen is a 45–55-kD glycoprotein that is a member of the immunoglobulin superfamily (8). Structurally, CD2 has two extracellular immunoglobulin-like domains, a transmembrane domain and a 116-amino acid cytoplasmic domain rich in proline residues (55). Early studies of CD2 function on T cells demonstrated that unique pairs of CD2-specific monoclonal antibodies (mAbs) can induce T cell proliferation in the absence of engagement of the CD3/T cell receptor (39). Subsequent studies by many laboratories have demonstrated that CD2 stimulation can induce IL-2 production (7), the activation of PLC-γ1 (46), PKC (2), and p21^{ras} (20), as well as increased tyrosine kinase activity (54), intracellular calcium (81), and intracellular cyclic AMP (26).

Analysis of CD2 in T cells has also suggested that CD2-mediated signaling is dependent on expression of the antigen-specific CD3/T cell receptor (9), which is consistent with reports that CD2 on T cells is physically associated with components of the T cell receptor complex (5, 10). CD2 is itself an adhesion molecule, capable of mediating T cell adhesion to other cells by binding to three different counter-receptors, CD58 (LFA-3), CD48, and CD59 (24, 35, 56). Furthermore, activation of T cells via the CD3/T cell receptor can also increase CD2-mediated adhesion to CD58, although the mechanism by which this occurs appears to be somewhat distinct from CD3/T cell receptor-mediated upregulation of integrin activity (23, 27). Structure-function analysis of the CD2 cytoplasmic domain has revealed regions of the cytoplasmic domain important for CD2-induced production of IL-2 as well as CD3/TCR-mediated regulation of CD2 adhesion to CD58 (6, 13, 14, 22, 27). With regard to its role as an integrin regulator, activating pairs of CD2-specific mAbs have been shown to result within minutes in increased adhesion of human peripheral T cells and CD2^+ human T cell lines to ICAM-1, FN, and laminin (40, 63, 78).

Phosphatidylinositol 3-kinase (PI 3-K) is an intracellular enzyme that phosphorylates the D-3 position of the inositol ring of phosphatidylinositol (PI), phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate. PI 3-K activity has been shown to be activated by a wide range of growth factors, oncogenes and other nonmitogenic stimuli (34). A wide variety of cellular functions appear to be dependent on PI 3-K, including growth factor-dependent mitogenesis, cytoskeletal rearrangements, glucose uptake, prevention of apoptosis, cytokine production, and endocytic trafficking (33, 34, 45, 83). The signaling pathways initiated by PI 3-K remain poorly defined, although recent studies have suggested that the lipid products generated by PI 3-K activity can activate certain isoforms of PKC in vitro (44, 73).

Structurally, PI 3-K consists of two subunits, p110 and p85. The p110 subunit is the catalytic subunit that contains a lipid kinase domain. The p85 subunit contains several amino acid motifs that allow for the association of PI 3-K with cytoplasmic receptors to form signaling complexes (47). The most well-described mode of association of PI 3-K with upstream signaling receptors occurs via tyrosine phosphorylation-dependent association of PI 3-K mediated by src-homology 2 (SH2) domains found on the p85 subunit (47). SH2-mediated binding of PI 3-K to multiple receptors has been demonstrated, including the platelet-derived growth factor (PDGF) receptor (30), the epidermal growth factor receptor (30), the major insulin receptor substrate IRS-1 (3), CD19 (76), and CD28 (45, 51, 74). The functional significance of this interaction has been demonstrated by the findings that: (a) SH2-mediated binding of p85 to receptors can activate PI 3-K (11, 43); (b) mutation of the SH2-binding motif in the PDGF-R inhibits PDGF-mediated mitogenesis (77) as well as PDGF-R internalization (33); and (c) mutation of the SH2-binding motif in the CD28 cytoplasmic domain inhibits CD28-mediated production of IL-2 (45). The p85 subunit also contains proline-rich sequences capable of mediating the binding of proteins containing SH3 domains (47). The SH3 domains of the intracellular tyrosine kinases abl, lck, fyn, and lyn
have all been shown to associate with this sequence in the p85 subunit (48, 52, 80). Finally, the p85 subunit itself contains an SH3 domain, and in vitro studies have demonstrated SH3-dependent binding of p85 to proline-rich sequences in the motor protein dynamin (19). Thus, the p85 subunit of PI 3-K contains several structural motifs that allow PI 3-K to be coupled to a multitude of cell surface and intracellular molecules.

In this study, we demonstrate a role for PI 3-K in the regulation of β1 integrin activity mediated by the integrin regulator CD2. We used DNA-mediated gene transfer to express this T cell integrin regulator in a non-T cell line, the myelomonocytic cell line HL60. Stimulation of CD2 expressed on HL60 cells was shown to result in increased β1 integrin-mediated adhesion of CD2+ transfectants to the β1 integrin ligands FN and VCAM-1. Increased β1 integrin activity mediated by CD2 stimulation but not PMA stimulation was completely inhibited by the PI 3-K inhibitor wortmannin. Furthermore, biochemical experiments demonstrated that while PI 3-K is associated with CD2 in both unstimulated and CD2-stimulated cells, PI 3-K activity associated with CD2 was detectable only in CD2-stimulated cells. Analysis of peripheral CD2+ T cells revealed that CD2 stimulation also resulted in PI 3-K-dependent upregulation of β1 integrin activity. Thus, these results not only demonstrate a role for PI 3-K in CD2-mediated up-regulation of β1 integrin activity but also highlight a potentially novel mode of association and activation of PI 3-K with CD2.

Materials and Methods

Cell Lines and Culture Reagents

The cell lines HL60, U937, and K562 were maintained in RPMI 1640 medium supplemented with 5% FCS (Sigma Chemical Co., St. Louis, MO), l-glutamine, and penicillin/streptomycin. During G418 selection after electroporation, the concentration of FCS was increased to 10%. G418 was purchased from GIBCO BRL (Gaithersburg, MD).

Human T cells were isolated by negative magnetic immunoselection from peripheral blood as previously described (29). The T cell populations obtained were routinely >98% CD3+CD2+ as assessed by flow cytometric analysis.

Antibodies and Other Reagents

The CD2-specific mAb 95-5-49 (53) (Dr. R. Gress, NIH, Bethesda, MD) was used as purified IgG for adhesion assays and as culture supernatants for screening of HL60 transfectants by flow cytometry. The following mAbs were used as dilutions of ascites fluid: CD2-specific mAbs 35.1 (American Type Culture Collection [ATCC], Rockville, MD) and 9-1 (provided by Dr. S. Y. Yang, Memorial Sloan Kettering Cancer Center, New York, NY), α2-specific mAb P1E6 and α3-specific mAb P1B5 (both GIBCO BRL), α4-specific mAbs L25 (38) (provided by Dr. P. Estess, Becton-Dickinson, Mountain View, CA) and NIH49D1 (provided by Dr. S. Shaw, NIH, Bethesda, MD), β1-specific mAb P4C10 (GIBCO BRL), activating β1-specific mAb P4G11 (provided by Dr. E. A. Wayner), and the LFA-1 α chain-specific mAb TS1/22 (ATCC). The α5-specific mAb B19 was provided by Dr. C. Damsky, University of California, San Francisco, CA) and the α1-specific mAb TS2/7 (ATCC) were used as culture supernatants. The LFA-1 β chain-specific mAb HM62 (Dr. S. Shaw, NIH, Bethesda, MD), the α6-specific mAb GoH-3 (Immunotech, Westbrook, ME), and the β1-specific mAb B-D15 (Biosource International, Camarillo, CA) were used as purified IgG. The p85-specific polyclonal antibody was obtained from Upstate Biotechnology (Lake Placid, NY). FN was purchased from the New York Blood Center (New York, NY). Chinese Hamster Ovary cells expressing a soluble form of VCAM-1 (49) (provided by Drs. B. Seed and C. Romeo, Massachusetts General Hospital, Boston, MA) was subcloned into the pMH-NeoH plasmid expression vector. This vector is identical to the pMH-Neo vector (25) (provided by Dr. B. Bierer, Dana-Farber Cancer Institute, Boston, MA), except that the HindIII site in the multiple cloning site has been eliminated by site-directed mutagenesis. The resulting construct (designated pMH-NeoH-CD2) was transfected into HL60 cells by electroporation. Briefly, 10^7 HL60 cells were washed once with Hepes buffer (20 mM Hepes pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM dextrose) and resuspended in 0.5 ml of ice-cold Hepes buffer containing 20 μg of uncut DNA. Electroporation was performed with a Bio-Rad Gene Pulsor set to 280V, 960 μF, resulting in a time constant of 11.9 ms. After electroporation, cells were allowed to sit on ice for 10 min before being diluted into 24 ml of RPMI/10% FCS and aliquoted into a single 24-well tissue culture plate. After 4 d of cell recovery, selection was initiated by the addition of RPMI/10% FCS medium containing 1 mg/ml G418 (0.5 ml/well). Every 4 d thereafter, 0.5 ml of medium was removed from each well and replaced with 0.5 ml of fresh G418-containing medium. Screening for CD2+ transfectants was performed 20-24 d after the electroporation.

To isolate CD2+ subclones, bulk transfectant populations were first enriched for CD2+ cells by passaging. Briefly, 6-well tissue culture plates were incubated overnight at 4°C with PBS containing 10 μg/ml of goat anti-mouse IgG (Organon Teknika, Malvern, PA). After the overnight incubation, plates were washed twice with PBS and incubated for 1-2 h at 37°C with PBS containing 2.5% BSA. Transfectants were washed once with PBS containing 0.5% human serum albumin (PBS/BSA) and then incubated in 1 ml of PBS/BSA containing 2-3 μg/ml of the CD2-specific mAb 95-5-49 for 30 min at 4°C. Cells were then washed twice with PBS/BSA, resuspended in 1.5 ml of PBS/BSA, added directly to the GAM-IgG-coated 6-well plates, and incubated for 2 h at 4°C. Nonadherent cells were removed by washing the plates three times with PBS, and RPMI/10% FCS medium containing 1 mg/ml G418 added to the wells to allow for expansion of the adherent cells. These bulk populations of transfectants were subsequently cloned by limiting dilution and screened for CD2 expression by flow cytometry. Representative subclones were chosen for further analysis.

Flow Cytometry

Single-color flow cytometry was performed as previously described (64) using saturating amounts of primary unlabeled mAb and detection with goat anti-mouse IgG FITC (Southern Biotechnology, Birmingham, AL), and 9-1. Samples were analyzed on a FACScan (Becton Dickinson).

Adhesion Assays

Adhesion assays were performed essentially as described before (42) with the exception of washing procedures. Briefly, 96-well microtiter plates (Costar No. 3596, Cambridge, MA) were incubated overnight at 4°C with the indicated concentrations of FN or VCAM-1 (diluted in PBS with Ca2+ and Mg2+) and unconjugated protein binding sites subsequently blocked with PBS/2.5% BSA. Each well contained 50,000 51Cr-labeled HL60 cells in a final volume of 0.1 ml PBS/BSA. For PMA activation, cells were added to wells containing 10 ng/ml of PMA. For CD2 stimulation, cells were added to wells containing 10 μg/ml of the CD2-specific mAb 95-5-49 and either a 1:1,000 dilution of the CD2-specific mAb 9-1 (for HL60 transfectants) or a 1:1,000 dilution of 9-1 (for peripheral T cells); this combination of CD2-specific mAbs has been previously demonstrated to induce T cell proliferation (65) and to result in increased β1- and β2-integrin-mediated adhesion of T cells to FN and laminin (40, 63). We refer to this pair of CD2-specific mAbs throughout this manuscript as the activating combination of CD2-specific mAbs. Integrin-specific antibodies and inhibitors (wortmannin and bisindolylmaleimide) were added to wells before the addition of cells and used at the concentrations indicated in the figure legends. After 1 h settling at 4°C, plates were quickly warmed to 37°C for 10 min.
Actin Polymerization

Changes in actin polymerization upon CD2 stimulation were assessed essentially as previously described (1). Briefly, duplicate samples of cells (1 × 10^6 cells/sample in a volume of 0.8 ml PBS/1% BSA) were either untreated, stimulated with 10 ng/ml PMA, or stimulated with the activating combination of CD2-specific mAbs for 10 min at 37°C in the absence or presence of 100 nM wortmannin. After stimulation, cells were permeabilized by adding 100 μl of a 1 mg/ml solution of lysophosphatidylcholine (Sigma) in 37% formalin and incubating for 10 min at 37°C. After permeabilization, 25 μl of a 6.6 μM stock of BODIPY-FL phallacidin (Molecular Probes, Eugene, OR) was added and the samples incubated for an additional 10 min at 37°C. Samples were then centrifuged, resuspended in ice-cold HBSS and analyzed on a FACScan. The mean channel fluorescence from each of the two duplicate samples was averaged and then normalized to the average mean channel fluorescence of unstimulated cells in the absence of wortmannin.

Results

β1 Integrin Expression and Function on HL60 Cells

The goal of this study was to develop a system where the integrin regulatory signaling properties of the CD2 antigen could be reconstituted by gene transfer in a CD2- cell line that also lacks expression of the CD3/CD2 beta complex. We reasoned that the recipient cell line to be used in these experiments needed to minimally fulfill the following three requirements. First, the cell line must lack expression of human CD2. Second, the cell line must express functional β1 integrins. Third, the β1 integrins expressed on this cell line must exhibit activation-dependent increases in function, as typically observed in peripheral human T cells and several T cell lines (40, 59, 62, 63). Increased β1 integrin function upon stimulation with the phorbol ester PMA was used as the criteria for fulfillment of this third requirement.

After screening multiple human non-T cell lines and mouse T cell hybridomas, several human cell lines were identified that fulfilled the above criteria. These included the erythroleukemia cell line K562, and myelomonocytic cell lines U937 and HL60. All three of these cell lines lack expression of CD2 (Fig. 1 and data not shown). The HL60 cell line was chosen for these studies because its pattern of β1 integrin expression most closely resembled that found on human peripheral blood T cells (40, 63): no detectable α1β1 and α2β1, low but detectable levels of α3β1 and α6β1 and clearly detectable levels of α4β1 and α5β1 (Fig. 1).
Stimulation with PMA results in rapid upregulation of \( \beta 1 \)-mediated adhesion of HL60 cells to FN. In A, binding of \( ^{51} \text{Cr} \)-labeled unstimulated (squares) and HL60 cells stimulated for 10 min at 37°C with 10 ng/ml PMA (circles) to various doses of FN was assessed as described in Materials and Methods. In B, the specificity of HL60 binding to FN (applied at 0.3 \( \mu \text{g/well} \)) was assessed by testing the ability of the following mAbs to inhibit the binding of unstimulated (shaded bars) and PMA-stimulated (solid bars) cells to FN: the \( \alpha 4 \)-specific mAb L25, the \( \alpha 5 \)-specific mAb BIIG2, the \( \beta 1 \)-specific mAb P4C10, the non-blocking \( \alpha 4 \)-specific mAb NIH49d-1, and the LFA-1 \( \beta \) chain-specific mAb MHM23. All mAbs were used at a concentration of 10 \( \mu \text{g/ml} \), except for BIIG2, which was used at a 1:10 dilution of culture supernatant. Binding to the negative control ECM protein collagen was <2% under all activation conditions tested. Data shown are representative of a minimum of five different experiments.

**Expression of CD2 on HL60 Cells**

**Results in CD2-stimulated Increases in HL60 \( \beta 1 \) Integrin Function**

To investigate the function of CD2 as an integrin regulator, DNA-mediated gene transfer was used to express CD2 in the HL60 cell line. Fig. 3 shows that transfection of a full-length human CD2 cDNA resulted in detectable levels of CD2 on the surface of G418-resistant transfectants. Levels of CD2 expression varied but several transfectants were obtained that expressed CD2 at levels comparable to that found on the CD2\(^+\) T cell line Jurkat (Fig. 3 and data not shown). We chose two transfectants for further analysis: HL60(CD2)-11.5, which expresses CD2 at levels comparable to Jurkat cells, and HL60(CD2)-23.9, which expresses lower levels of CD2 than either the 11.5 transfectant or Jurkat cells. All transfectants tested had levels of \( \beta 1 \), \( \alpha 4 \), and \( \alpha 5 \) comparable to untransfected HL60 cells (Fig. 3 and data not shown).

Unique pairs of CD2-specific mAbs have been shown to activate human T cells, as measured by cellular proliferation, cytokine production and increases in integrin-mediated adhesion (7, 39, 40, 63, 78). We have previously demonstrated that incubation of either peripheral human T cells or human T cell lines, such as Jurkat, with the combination of the CD2-specific mAbs 95-5-49 and 9-1 results within minutes in increased adhesion to various integrin ligands and counter-receptors (40, 63). Stimulation of CD2\(^+\) HL60 transfectants with this activating combination of CD2-specific mAbs also resulted in increased HL60 adhesion to FN (Fig. 4). For the 11.5 transfectant, which expresses CD2 at levels comparable to the Jurkat T cell line, the integrin adhesion regulatory signal provided by CD2 stimulation was as effective as PMA stimulation at all doses of FN tested. The CD2 adhesion response was pre-
CD2 expression on CD2+ HL60 transfectants. Single color flow cytometric analysis was performed as described in Materials and Methods. Binding of the CD2-specific mAb 95-5-49 (left column) and the β1-specific mAb B1-D5 (right column) is shown as filled histograms in each panel. Open histograms represent negative control staining of cells in the absence of a primary antibody and is shown in each panel for ease of comparison. HL60(CD2)-11.5 and HL60(CD2)-23.9 are two representative transfectants of HL60 cells expressing high (11.5) and intermediate (23.9) levels of CD2. CD2 and β1 integrin expression on the CD2+ human T cell line Jurkat is shown to illustrate the level of CD2 expression on HL60 transfectants compared to a human cell that normally expresses CD2.

Although PMA stimulation results in increased binding of HL60 cells to FN via both the α4β1 and α5β1 integrins (Figs. 2B and 5), similar antibody blocking studies using CD2-stimulated CD2+ HL60 transfectants suggest that CD2 stimulation results in preferential upregulation of α5β1 relative to α4β1. Thus, while an α5-specific mAb only partially inhibits the binding of PMA-stimulated CD2+ transfectants to FN, the same α5-specific mAb completely inhibits the binding of CD2-stimulated transfectants to FN (Fig. 5).

To determine whether CD2 stimulation also results in increased α4β1-mediated adhesion, the binding of CD2+ HL60 transfectants to the α4β1 cell surface counter-receptor VCAM-1 was assessed. Fig. 6 shows that while unstimulated HL60 cells and CD2+ HL60 transfectants fail to bind to various doses of affinity-purified VCAM-1, PMA-stimulation increases the binding to VCAM-1 of all three cell lines tested. For the 11.5 transfectant that expresses levels of CD2 comparable to human T cells, CD2 stimulation results in increased adhesion to VCAM-1 comparable to that seen after PMA-stimulation. Interestingly, the 23.9 transfectant that expresses lower levels of CD2 does not show increased adhesion to VCAM-1 after CD2 stimulation except at the highest dose of VCAM-1 tested (Fig. 6). Since the level of α4β1 integrin expression on the 11.5 and...
Figure 5. Binding of CD2-stimulated HL60 transfectants to FN is inhibited by α5- and β1-specific mAbs. Binding of unstimulated (open bars), PMA-stimulated (shaded bars), and CD2-stimulated (solid bars) HL60(CD2)-11.5 transfectants to FN (applied at 0.3 μg/well) was assessed in the presence of the following mAbs: α4-specific mAb L25, α5-specific mAb BIIG2, β1-specific mAb P4C10, non-blocking α4-specific mAb NIH49d-1, and the LFA-1 α-chain-specific mAb TS1/22. MAbs were used at the concentrations indicated in the legend to Fig. 2. PMA was used at a concentration of 10 ng/ml, and the activating combination of CD2-specific mAbs consisted of 10 μg/ml of 95-5-49 and a 1:6,000 dilution of 9-1. Activation was for 10 min at 37°C. Binding to the negative control ECM protein collagen was <3% under all activation conditions tested. Data are representative of a minimum of four different experiments.

Figure 6. Antibody stimulation of CD2 results in upregulation of adhesion of CD2+ HL60 transfectants to the α4β1 counter-receptor VCAM-1. Binding of HL60 cells (left), and the CD2+ HL60 transfectants HL60(CD2)-11.5 (middle) and HL60(CD2)-23.9 (right) to various doses of VCAM-1 was assessed in the presence of: no stimulus (circles), 10 ng/ml PMA (squares), or an activating combination of CD2-specific mAbs (triangles). Cells were activated for 10 min at 37°C before washing away the nonadherent cells. Binding to the negative control ECM protein collagen was <3% under all activation conditions tested. Data are representative of a minimum of three different experiments.

The PI3-K Inhibitor Wortmannin Inhibits CD2 Adhesion Regulation in HL60 Transfectants

The fungal metabolite wortmannin has recently been shown to be a highly specific inhibitor of PI 3-K when used at nanomolar concentrations (15, 50, 57). Fig. 7A shows that treatment of the 11.5 transfectant with 100 nM of wortmannin completely inhibits the ability of the activating combination of CD2-specific mAbs to upregulate β1 integrin-mediated adhesion to FN. The specificity of the inhibitory effects for CD2-mediated upregulation of integrin function is suggested by the minimal effects of wortmannin on adhesion induced by PMA stimulation (Fig. 7A) or the activating β1-specific mAb P4G11 (data not shown). Dose response studies indicate that the half-maximal concentration (IC50) of wortmannin that inhibits CD2-induced increases in CD2+ HL60 transfectant adhesion to FN is in the range of 30–40 nM (Fig. 7B). This IC50 value is consistent with concentrations of wortmannin found to specifically inhibit PI 3-K-dependent cellular responses and PI 3-K activity in other systems (15, 57). Thus, these results suggest that the signal transduced by CD2 stimulation that results in upregulation of β1 integrin function in HL60 transfectants is dependent on PI 3-K.

Constitutive Association of CD2 with PI 3-K

The ability of wortmannin to inhibit CD2-induced increases in β1 integrin functional activity on CD2+ HL60 transfectants suggested an association between the CD2 antigen and PI 3-K. Since PI 3-K has not previously been shown to be associated with CD2, we tested this hypothesis by immunoprecipitating the CD2 antigen from unstimulated, CD2-stimulated, and PMA-stimulated CD2+ HL60 transfectants and assessing the presence of the p85 subunit of PI 3-K in CD2 immunoprecipitates by immunoblotting. Fig. 8 demonstrates that the p85 subunit of PI 3-K can be detected in CD2 immunoprecipitates of CD2+ HL60 trans-
Figure 7. CD2-mediated upregulation of adhesion of CD2+ HL60 transfectants to FN is completely inhibited by the PI 3-K inhibitor wortmannin. (A) Binding of HL60(CD2)-11.5 cells to various doses of FN was assessed in the presence of no stimulus (circles), 10 ng/ml PMA (triangles), or an activating combination of CD2-specific mAbs (squares) in the absence (solid symbols) or continuous presence (open symbols) of 100 nM wortmannin. Cells were activated for 10 min at 37°C before washing away the nonadherent cells. Data are representative of a minimum of seven different experiments. (B) The effect of various doses of wortmannin on the adhesion of HL60(CD2)-11.5 cells to FN (applied at 1 μg/well) after no stimulation (triangles), PMA-stimulation (diamonds) and CD2-stimulation (squares) was assessed. Activation conditions were identical to those used in A. Data are representative of a minimum of four different experiments.

Figure 8. Constitutive association of PI 3-K with CD2. HL60 (CD2)-11.5 cells (10^7 cells/stimulation condition) were either unstimulated (U), CD2-stimulated for 0, 0.5, 1, 2, 5, and 10 min, or PMA-stimulated for 10 min, lysed in PI 3-K lysis buffer, and immunoprecipitated with the CD2-specific mAb 35.1 as described in Materials and Methods. Unstimulated HL60 cells were also prepared in a similar manner. 8 x 10^6 cell equivalents were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and immunoblotted with an antibody specific for the p85 subunit of PI 3-K. Data are representative of a minimum of four different experiments. Similar results were obtained when the lysates were prepared using 1x modified RIPA buffer (see Materials and Methods).

Two important points should be noted regarding the association of CD2 with p85. First, p85 is associated with CD2 expressed on unstimulated CD2+ HL60 transfectants. Second, stimulation of transfectants with the activating combination of CD2-specific mAbs or PMA does not increase the amount of p85 associated with CD2. Thus, the association of PI 3-K with CD2 appears to be distinct from the well-described mode of SH2-mediated binding of p85 to many other cell surface receptors, where PI 3-K association is dependent on activation-dependent tyrosine phosphorylation of the receptor's cytoplasmic domain (47). These results indicate that PI 3-K associates with CD2 expressed in HL60 cells in a constitutive activation-independent fashion.

CD2 Stimulation Results in Activation of PI 3-K

To more definitively address the significance of the CD2-associated PI 3-K detected in immunoblotting experiments, we directly tested PI 3-K enzymatic activity in immunoprecipitates of CD2 isolated from unstimulated and CD2-stimulated HL60(CD2)-11.5 transfectants. PI 3-K activity in CD2 immunoprecipitates was assessed by testing the ability of CD2 immunoprecipitates to phosphorylate
Figure 9. CD2 stimulation of HL60(CD2)-11.5 cells results in activation of CD2-associated PI 3-K. HL60(CD2)-11.5 cells (2 x 10^7 cells/sample) were either unstimulated or stimulated with the activating combination of CD2-specific mAbs for 10 min at 37°C, lysed in PI 3-K lysis buffer, and then immunoprecipitated with either the CD2-specific mAb 35.1 (left) or a p85-specific antibody (right). Samples were then tested for PI 3-K activity as described in Materials and Methods, the phosphatidylinositol (PI) separated by thin layer chromatography and the labeled lipid products visualized by autoradiography.

Although PI 3-K is found to be associated constitutively with CD2 in unstimulated HL60 transfectants, there is minimal PI 3-K activity in CD2 immunoprecipitates from unstimulated cells (Fig. 9). In contrast, stimulation of CD2+ HL60 transfectants with the activating combination of CD2-specific mAbs induces PI 3-K activity in CD2 immunoprecipitates. Control immunoprecipitates using a p85-specific antibody demonstrate that PI 3-K activity can be detected in both unstimulated and CD2-stimulated cells. Thus, even though CD2 stimulation fails to increase the amount of PI 3-K associated with CD2, stimulation with CD2-specific mAbs does result in increased activity of the associated PI 3-K. These results, coupled with the potent inhibitory effects of wortmannin in our adhesion assays, support the hypothesis that CD2-mediated regulation of β1 integrin function occurs via a PI 3-K-dependent pathway.

Partial Inhibition of CD2-mediated Integrin Upregulation by PKC Inhibitors

Although the exact intracellular responses initiated by PI 3-K activation remain poorly defined, several studies have recently suggested that the lipid products generated by PI 3-K activity can activate several isoforms of PKC (44, 73). To begin to assess potential downstream effects of PI 3-K-dependent upregulation of integrin function mediated by CD2 stimulation, we tested the ability of the PKC inhibitor bisindolylmaleimide to inhibit CD2-induced increases in binding to FN. Fig. 10 shows that treatment of the CD2+ 11.5 transfectants with bisindolylmaleimide inhibited adhesion induced by the activating combination of CD2-specific mAbs (Fig. 10). However, at concentrations of bisindolylmaleimide (3 and 10 μM) that completely inhibited the binding of PMA-stimulated transfectants to FN, inhibition of binding of CD2-stimulated cells to FN was only partial (Fig. 10). As expected, bisindolylmaleimide had no effect on adhesion induced by the activating β1-specific mAb P4G11 (Fig. 10).
CD2 Stimulation Results in Wortmannin-sensitive Increases in F-actin Content in HL60 Transfectants

Activation of PI 3-K has also been implicated in alterations of the cytoskeleton (34, 58). Since such cytoskeletal rearrangements could have an effect on cell adhesion, we determined whether CD2 stimulation of CD2⁺ HL60 transfectants resulted in actin polymerization, as measured by increased staining of the F-actin dye BODIPY FL-phallacidin. Fig. 11 shows that stimulation of 11.5 transfectants with the activating combination of CD2-specific mAbs results in increased F-actin content when compared to unstimulated cells. Interestingly, PMA-stimulation of the same cells failed to result in actin polymerization, suggesting that PKC activation is not involved in this cytoskeletal rearrangement. A role for PI 3-K in CD2-mediated actin polymerization is suggested by the ability of wortmannin to inhibit the increase in F-actin content observed in CD2⁺ HL60 transfectants upon stimulation with CD2-specific mAbs (Fig. 11).

CD2 Stimulation of Peripheral Blood T Cells Results in PI 3-K-dependent Upregulation of β1 Integrin Activity

To confirm that PI 3-K has a similar role in CD2 adhesion regulation in cells that normally express CD2, we assessed CD2-associated PI 3-K activity in CD2⁺ peripheral blood T cells. We have previously demonstrated that stimulation of peripheral T cells with the activating combination of CD2-specific mAbs results in increased β1 integrin-mediated adhesion of T cells to FN and laminin (63). Fig. 12 shows that, similar to what was observed with CD2⁺ HL60 transfectants, wortmannin inhibits the increased adhesion of human T cells to FN that occurs after CD2 stimulation, but not PMA stimulation. CD2 stimulation also resulted in increased actin polymerization in human T cells that could be inhibited by wortmannin (data not shown).

Immunoblotting analysis also demonstrates that CD2 in human T cells is constitutively associated with the p85 subunit of PI 3-K and CD2 stimulation does not result in increased p85 association with CD2 (Fig. 13 A). In vitro analysis of the activity of CD2-associated PI 3-K does reveal one difference between CD2 expressed on human T cells and CD2 expressed on HL60 cells. Compared to CD2⁺ HL60 transfectants, there is a higher basal level of PI 3-K activity in CD2 immunoprecipitates isolated from unstimulated human T cells (Fig. 13 B). However, similar to what was observed in HL60 transfectants, CD2 stimulation of human T cells also results in increased activity of the CD2-associated PI 3-K (Fig. 13 B). In multiple experiments, CD2-stimulation consistently resulted in a 1.5-2-fold increase in the activity of the associated PI 3-K, as assessed by quantitative analysis using a phosphorimager.

Discussion

Using DNA-mediated gene transfer, we have been able to reconstitute the integrin regulatory activity of the human T cell antigen CD2 in a non-T cell line, the myelomonocytic cell line HL60. Stimulation with an activating combination of CD2-specific mAbs was able to increase binding of HL60 cells to FN and VCAM-1 to levels comparable to that seen with PMA-stimulation. Further analysis of these transfectants suggest a critical role for PI 3-K in the regulation of integrin activity by CD2. First, CD2-stimulated increases in β1 integrin activity could be completely inhibited by the PI 3-K inhibitor wortmannin. Second, PI 3-K could be detected in CD2 immunoprecipitates prepared from both unstimulated and CD2-stimulated HL60 transfectants.
PI 3-K is associated with CD2 and activated by CD2 stimulation of T cells. 

Figure 13. PI 3-K is associated with CD2 and activated by CD2 stimulation of T cells. (Left) Unstimulated T cells or T cells stimulated with the activating combination of CD2-specific mAbs for 10 min were lysed in PI 3-K lysis buffer, and immunoprecipitated with the CD2-specific mAb 35.1 as described in Materials and Methods. Immunoblotting for detection of the p85 subunit was performed as described in Fig. 8 legend. (Right) T cells were either unstimulated or stimulated with the activating combination of CD2-specific mAbs for 10 min at 37°C, lysed in PI 3-K lysis buffer, and then immunoprecipitated with the CD2-specific mAb 35.1. Samples were then tested for PI 3-K activity as described in Fig. 9 legend. Quantitative analysis using a phosphorimager revealed that CD2 stimulation of human T cells results in a 1.7-fold increase in radiolabeled PIP when compared to unstimulated T cells. Data are representative of a minimum of four experiments.

These studies provide new evidence that PI 3-K is a critical intracellular mediator involved in integrin upregulation mediated by CD2 stimulation. We propose the following model of CD2-mediated signaling that leads to integrin upregulation that is consistent with the results presented in this study. It is clear from our immunoblotting experiments that PI 3-K is constitutively associated with CD2 in unstimulated cells but that the associated PI 3-K is relatively inactive. Stimulation of CD2 with the activating combination of CD2-specific mAbs results in activation of the already associated PI 3-K but does not induce increased association of additional PI 3-K. Activation of PI 3-K by CD2 stimulation is required for CD2-mediated increases in β1 integrin activity and leads to two downstream, independent PI 3-K-dependent responses: activation of PKC and actin polymerization. This is based on the ability of the PKC inhibitor bisindolylmaleimide to partially inhibit CD2-dependent increases in β1 integrin function in our HL60 transfectants, as well as our finding that CD2 stimulation results in increased actin polymerization that can be inhibited by wortmannin. We propose that PI 3-K-dependent actin polymerization and PKC activation are independent downstream events, since PMA stimulation fails to increase F-actin content in our CD2+ HL60 transfectants. Our findings on actin polymerization are consistent with other studies that have clearly implicated PI 3-K in regulation of the actin cytoskeleton (34, 58). Since CD2 stimulation has previously been shown to result in PKC activation (2), an alternative possibility is that CD2 stimulation results in an independent pathway of up-regulation of β1 integrin function that involves direct activation of PKC independent of PI 3-K activation. However, our model proposing that PKC activation is downstream of PI 3-K activation in the CD2 integrin regulatory pathway is consistent with the fact that: (a) the PKC inhibitor bisindolylmaleimide is much less effective than wortmannin in inhibiting CD2-dependent increases in integrin function; and (b) studies in other systems have demonstrated that the lipid products generated by PI 3-K activity can activate certain PKC isoforms in vitro (44, 73).

Previous studies of T cell integrin regulators have not implicated PI 3-K in this regulatory event, although other intracellular mediators such as tyrosine kinases, protein kinase A, and pertussis toxin-sensitive G proteins have been implicated (4, 12, 17, 61). However, studies of mast cell adhesion to FN have recently shown that treatment of mast cells with stem cell factor, the ligand for the c-kit growth factor receptor, can up-regulate β1 integrin-mediated mast cell adhesion to FN (16, 36). Furthermore, the c-kit receptor associates with PI 3-K in a tyrosine phosphorylation-dependent manner and integrin upregulation on mast cells induced by stem cell factor has recently been shown to be dependent on c-kit-associated PI 3-K activity (57). Thus, it is now clear that an additional cellular response mediated by PI 3-K is alterations in cell adhesion. T cell integrin function has recently been shown to be upregulated by hepatocyte growth factor (HGF), and this increase in activity correlated with HGF-induced increases in actin polymerization (1). Although the HGF receptor, the c-met oncogene, does not appear to be expressed in human T cells (1), HGF binding to the c-met receptor in other cell types has been shown to result in increased tyrosine kinase activity, as well as tyrosine phosphorylation-dependent association and activation of PI 3-K (21). It will be important in the future to determine if other growth factor receptors that have been shown to associate with PI 3-K might also be involved in regulating integrin functional activity.

We have also previously shown that antibody crosslinking of the CD28 antigen expressed on human T cells results in increased β1 and β2 integrin function (40, 61). Interestingly, recent studies of CD28 function have revealed that CD28 is associated with PI 3-K in a tyrosine phosphorylation-dependent manner (45, 51, 74), and PI 3-K associated with CD28 is necessary for CD28-mediated production of IL-2 (45). The ability of wortmannin to inhibit CD28-induced increases in β1-mediated adhesion to FN suggests a role for PI 3-K in the adhesion regulatory properties of this receptor as well (T. Zell, S. W. Hunt III, J. L. Mobley, and Y. Shimizu, manuscript in preparation). We have also recently found that PI 3-K associates in a tyrosine phosphorylation-dependent manner with the CD7 antigen and that wortmannin can completely inhibit CD7-induced increases in β1 integrin activity on human T cells (A. S. H. Chan, J. L. Mobley, and Y. Shimizu, manuscript in preparation). Since the submission of this manuscript,
two studies provide evidence that regulation of integrin function induced by chemokines may also be dependent on PI 3-K. In one study, treatment of T cells with RANTES, a potent T cell chemotaxant, was found to result in increased PI 3-K activity (75). RANTES-induced T cell chemotaxis was also found to be inhibited by wortmannin (75). In the second study, a novel p110 PI 3-K subunit was identified that could be activated in vitro by G proteins (69). This is an intriguing finding, since treatment of T cells with G protein inhibitors, such as pertussis toxin, was found to inhibit activation-induced increases in integrin activity that occur during lymphocyte interactions with endothelial cells in vivo (4). Thus, there is increasing evidence to suggest that the activity of other integrin regulators expressed on T cells may also be dependent on the induction of associated PI 3-K activity.

Our studies with CD2-induced adhesion regulation in normal CD2+ T cells are consistent with our findings using CD2+ HL60 transfectants. CD2-induced increases in β1 integrin activity and actin polymerization in T cells can be inhibited by wortmannin. PI 3-K is associated with CD2 in T cells and CD2 stimulation increases CD2-associated PI 3-K activity. However, two differences were observed between T cells and HL60 cells with regard to CD2 and PI 3-K. First, wortmannin did not completely inhibit CD2-induced increases in adhesion of T cells to FN at doses that completely inhibited CD2-induced adhesion of HL60 transfectants. This suggests that CD2 may also use a PI 3-K-independent signaling pathway in human T cells for regulating β1 integrin activity. Second, there was a greater level of basal PI 3-K activity in CD2 immunoprecipitates from unstimulated T cells compared to unstimulated HL60 cells. The reason for these differences is not clear at present, although the reported physical and functional association between CD2 and the CD3/T cell receptor complex (5, 10) suggests that the presence of the T cell receptor in human T cells may be an important factor. This is currently being investigated.

PI 3-K associates with the c-kit receptor, CD28, and many other cell surface receptors via the SH2 domain of the p85 subunit of PI 3-K with a specific phosphotyrosine-containing amino acid motif in the receptor’s cytoplasmic domain (47, 66). Thus, such SH2-dependent association of PI 3-K requires that receptor activation induce some associated tyrosine kinase activity. The association of CD2 with PI 3-K in HL60 cells does not appear to be mediated by the p85 SH2 domain. First, the CD2 cytoplasmic domain lacks any tyrosine residues (55). Second, our results demonstrate that PI 3-K is associated with CD2 in unstimulated cells and CD2 stimulation fails to increase the amount of associated PI 3-K. Therefore, the mechanism by which CD2 associates with PI 3-K does not involve tyrosine phosphorylation-dependent association of p85 with CD2.

Although the mode of association of CD2 with PI 3-K remains undefined, several lines of evidence suggest that PI 3-K may associate with CD2 via an SH3-dependent interaction. First, the p85 subunit of PI 3-K possesses an SH3 domain (47) and an SH3-dependent interaction of PI 3-K with the motor protein dynamin has been demonstrated (19). Second, SH3 domains have been shown to mediate protein–protein interactions by binding to proline-rich sequences (47). Third, the CD2 cytoplasmic domain is rich in proline residues that might be capable of mediating SH3 domain interactions (55). The role of two repeated PPGGR motifs in the CD2 cytoplasmic domain in integrin regulation is of particular interest, since deletion of either or both of these motifs has been shown to impair CD2-mediated production of IL-2 and increases in cyclic AMP (6). We are currently using our system to determine the effects of mutations in the CD2 cytoplasmic domain on association with PI 3-K as well as on CD2-induced upregulation of integrin activity. These ongoing studies will allow us to precisely map the region in the CD2 cytoplasmic domain mediating the association with PI 3-K and whether this association is dependent on the p85 SH3 domain. The functional consequences of an SH3-mediated interaction of PI 3-K with CD2 as compared to the more well-characterized SH2-dependent interaction could be quite significant. Since the p85 subunit contains SH2 domains and an SH3 domain, association of CD2 with PI 3-K via the SH3 domain would allow the CD2/PI 3-K complex to potentially associate with other intracellular molecules via the free SH2 domains in the p85 subunit.

Binding of the SH3 domains of the lyn and fyn tyrosine kinases to a proline-rich region in the p85 subunit of PI 3-K has recently been shown to result in increased PI 3-K activity (48). These results suggest that SH3 domain binding to these proline-rich sequences in p85 is sufficient by itself to activate PI 3-K, although the mechanism by which this occurs is unknown. This is distinct from our findings of CD2 association with PI 3-K, since the p85 subunit can be found to be associated with CD2 in unstimulated cells but is enzymatically inactive. Thus, both the mode of association of PI 3-K with CD2 as well as the regulation of PI 3-K activity by CD2 may be distinct from other previously described PI 3-K–associated receptor systems.

In summary, we have demonstrated a role for PI 3-K in the ability of the CD2 antigen to function as an integrin regulator. The association of CD2 with PI 3-K does not occur via a tyrosine phosphorylation-dependent mechanism and may involve a novel mode of protein–protein interaction. Furthermore, our ability to reconstitute CD2 integrin regulatory activity in a non-T cell line suggests that T cells and HL60 cells share common intracellular pathways involved in integrin regulation. This is consistent with other studies demonstrating reconstitution of the integrin regulatory activity of the f-met-leu-phe receptor from neutrophils in a lymphoma cell line (28). Finally, it should be possible to use this system to systematically analyze the structural requirements for the function of CD2 as well as other integrin regulators. Such studies should lead to a determination of whether PI 3-K represents a common intracellular mediator involved in regulation of integrin functional activity.

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