Surface CD3 expression proceeds through both myosin regulatory light chain 9 (MYL9)-dependent and MYL9-independent pathways in Jurkat cells

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Abstract

CD3 is a complex of polypeptides which form part of the T cell receptor. Normal human peripheral pan T cells, express not only CD3, but the mRNA for myosin regulatory light chains MYL9, MYL12A, and MYL12B are also significantly expressed. In the Jurkat wild strain, an acute T cell leukemia cell line, CD3 on the surface and MYL9 mRNA are not expressed, while both MYL12A mRNA and MYL12B mRNA are expressed. Jurkat-I, a new clone was established by the transfection of the MYL9 gene into the Jurkat wild strain. As a result, the level of CD3 expressed on the surface of Jurkat-I cells was significantly higher than those in the Jurkat wild strain. Phorbol 12-myristate 13-acetate increased the surface CD3 levels in Jurkat wild strain cells without resulting in MYL9 gene expression, indicating that protein kinase C is partially involved in the expression of CD3 on the surface. These results suggest that surface CD3 expression proceeds through both MYL9-dependent and MYL9-independent pathways (i.e. the protein kinase C-dependent pathway) in Jurkat cells.

Key words: myosin regulatory light chain 9, CD3, Jurkat cell, T cell

Introduction

Most myosin subtypes belong to class II and, together with actin, make up the major contractile proteins of cardiac, skeletal and smooth muscle cells. Myosin II molecules are also present in all non-muscle eukaryotic cells. Like muscle myosin II, non-muscle myosin II (NM II) molecules are comprised of three pairs of peptides: two heavy chains of 230 kDa, two 20 kDa regulatory light chains (RLCs) that regulate NM II activity and two 17 kDa essential light chains (ELCs) that stabilize the...
heavy chain structure (Ikebe, 1989). Three different genes in mammalian cells (myosin heavy chain 9 or MYH9, MYH10, and MYH14) encode the NM II heavy chain (NMHC II) proteins (NMHC IIA, NMHC IIB, and NMHC IIC, respectively) (Conti et al., 2008). The regulation of NMHC IIA, NMHC IIB, and NMHC IIC as well as smooth muscle myosin depends on the reversible phosphorylation of the RLC on Ser19 or Thr18 (Bresnick, 1999; Vicente-Manzanares et al., 2009). Multiple kinases, including myosin light chain kinase (MLCK), Rho-associated, coiled-coil forming protein kinase (ROCK) and myotonic dystrophy kinase-related CDC42-binding kinase (MRCK) can phosphorylate the regulatory light chain (RLC) of NM II on Ser19 or on Thr18 and Ser19 to activate it (Matsumura, 2005; Tan et al., 2008). Protein kinase C (PKC) phosphorylates RLCs on Ser1, Ser2, and Thr9, and decreases the activity of NM II (Nishikawa et al., 1984; Vicente-Manzanares et al., 2009). Twenty kDa RLC is phosphorylated by MLCK in the presence of calcium and calmodulin and increases actin-activated ATPase activities of myosins of both smooth muscle (Adelstein et al., 1980; Somlyo et al., 1994) and non-muscle cells such as platelets (Ikebe, 1989; Higashihara et al., 1991) and red blood cells (Higashihara et al., 1989). RLC phosphorylation of cytoplasmic myosin has been implicated in cytokinesis, receptor capping, and cell locomotion (Bourguignon et al., 1981; Yamakita et al., 1994; Matsumura et al., 1998).

We previously identified three kinds of myosin regulatory light chains (MLC-2A, MLC-2B, and MLC-2C) from human blood cells (Watanabe et al., 2001; Higashihara et al., 2008). Presently MLC-2A, MLC-2B, and MLC-2C have been named MYL12B, MYL12A, and MYL9, respectively. Both MYL12A and MYL12B mRNA are constitutively conserved in all hematopoietic cells analyzed so far (Higashihara et al., 2008). MYL9 mRNA was observed only in monocytic cell lines (U937 and A-THP-1) and erythroid cell lines (K562 and HEL). MYL12A, MYL12B, and MYL9 genes are highly conserved in their gene structures and amino acid sequences (Higashihara et al., 2008). The amino acid sequence of the MYL9 gene is apparently almost the same as that of the human vascular smooth muscle LC-20 (Higashihara et al., 2008).

It was recently suggested that the MYL9 gene is a direct target of RUNX1 and provides a mechanism for decreased platelet MYL9 expression, myosin light chain (MLC) phosphorylation, thrombocytopenia, and platelet dysfunction associated with RUNX1 mutations (Jalagadugula et al., 2010). However, the functions of MYL9 in blood cells have not yet been clarified.

The CD3 complex of polypeptides forming part of the T cell receptor (TCR), consists of four different chains: γ, δ, ε and ζ. CD3 antigen is a crucial molecule in T cell signal transduction (Marrack et al., 1987; Raulet, 1989). Although its expression on the cell surface is constitutive, dynamic regulation of TCR-CD3 level is probably the most important mechanism allowing T cells to calibrate their response to different levels of stimuli (Wegener et al., 1992).

In all peripheral T cells and mature thymocytes, CD3 antigen has been significantly expressed on the cell surface. On the other hand, CD3 has not been expressed by Jurkat wild strain cells, the acute T cell leukemia cell line. Several levels of CD3 surface expressions were observed by limiting dilutions of Jurkat E6-1 cultures (Jóźwik et al., 2004). MYH9 is dominant in T cells and pairs with regulatory MLCs to form a complex known as NM IIA (Jacobelli et al., 2004). NM IIA is rapidly activated upon TCR engagement and its activity is essential for the centripetal movement of TCR microclusters (Ilani et al., 2009).

In this study using MYL9 gene transfect methods, we report that the expression of surface CD3 in Jurkat cells has a causal relationship with the expression of the MYL9 gene.
Materials and Methods

Cell lines and cell cultures

Cultures of the Jurkat wild strain cell line were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Tokyo, Japan). The culture of the Jurkat clone E6-1 was purchased from the American Type Culture Collection (ATCC®) (Manassas, VA, USA). All cell lines were cultured in RPMI 1640 containing 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin, and 2 mM L-glutamine.

Chemical

Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich (St.Louis, MO, USA). PMA was dissolved in dimethylsulfoxide (DMSO). Antibiotic A23187 was purchased from Enzo Life Sciences International Inc. (Plymouth Meeting, PA, USA).

cDNA of human normal peripheral blood cells

Normal peripheral blood Pan T Cells cDNA (cDNA-PB002), Platelets cDNA (cDNA-PB015), B Cells CD19+ cDNA (cDNA-PB003), and Monocytes CD14+ cDNA (cDNA-PB004) were purchased from AllCells (Emeryville, CA, USA). In the reverse transcription-polymerase chain reaction (RT-PCR), the 275 bps product that existed after the reactions were run using 2 μl of cDNA with β-Actin Primers, after 25 cycles.

Oligonucleotides and polymerase chain reaction (PCR)

Oligonucleotides were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). For PCR we used Premix Taq® (TaKaRa Taq™ Version) (TaKaRa, Shiga, Japan) and standard PCR reaction mixtures with 25 pmole each of forward and reverse primers in a 50 μl cocktail. All specific primers used are listed in Table 1.
Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using ISOGEN™ (Nippon Gene, Co., Ltd, Tokyo, Japan). First strand cDNA was synthesized using dNTP Mix (Life Technologies, Carlsbad, CA, USA), RNase inhibitor (Toyobo, Osaka, Japan), random primer (Hokkaido System Science, Sapporo, Japan), and Reverse Transcriptase M-MLV (RNase H free) (Takara, Shiga, Japan). The reaction was carried out in 20 μl cocktail. Samples were incubated at 37°C for 60 min, and reactions were heated at 70°C for 10 min.

Each reaction mixture contained template cDNA 2.0 μl. The reaction condition of MYL9, MYL12A, and MYL12B was settled as follows: initial denature at 94°C for 30 sec, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 20 sec, and the final extension at 72°C for 7 min. The reaction condition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was as follows: initial denature at 94°C for 30 sec, 30 cycles of 94°C for 45 sec, 54°C for 45 sec, 72°C for 1 min, and the final extension at 72°C for 5 min. PCR-amplified products were gel-purified with DNA Molecular Weight Marker XIV (100–1,500 bp) (Roche, Mannheim, Germany).

Flow cytometry and monoclonal antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-IgG monoclonal antibody, phycoerythrin (PE)-conjugated anti-IgG monoclonal antibody, FITC-conjugated anti-CD3ε monoclonal antibody; anti-CD3 (FITC), PE-conjugated anti-CD3ε monoclonal antibody; anti-CD3(PE), and IntraPrep™ were purchased from Beckman Coulter (Marseille Cedex 9, France). Cytometric analysis was performed on the FACS (Beckman Coulter) at the Department of Clinical Laboratory, Kitasato University Hospital. Surface CD3 expression was measured by staining with anti-CD3 (FITC) or anti-CD3 (PE). CD3 (FITC) and CD3 (PE) indicate CD3 positivity (%) detected by anti-CD3 (FITC) and anti-CD3 (PE), respectively. IntraPrep™ consists of two ready-to-use reagents, which induce permeability in the cytoplasmic membrane of leucocytes for the demonstration of intracellular antigenic determinants by means of monoclonal fluorescent antibodies. The expression levels of CD3 (FITC) in the cytoplasm were determined by flow cytometry (FCM) after the Jurkat wild strain and Jurkat-I cultures were permeated with IntraPrep™. The positivity was shown in both percentage (%) and mean fluorescence intensity (MFI) per cell.

Production of the Jurkat-I culture by MYL9 gene transfection

RPMI 1640, FBS (-) 485.3 μl, pCI-neo Mammalian Expression Vector/MLC-2C (Promega, Madison, WI, USA) (1.51 μg/μl) 2.65 μl and TransFast™ Transfection Reagent (Promega) (1 μg/μl) 12 μl were combined and incubated for 20 min at room temperature. The mixture was added to the culture of Jurkat wild strain cells (2 × 10^6 cells/mL) 500 μl. The mixture was plated in a 6-well plate, re-suspended in 5 ml of 10% FCS + RPMI 1640 and incubated for 48 h at 37°C. The clones were selected by Antibiotic G-418 Sulfate Solution (Promega) (1.0 mg/ml). The expression of MYL9 mRNA was observed in 6 clones (A, E, F, G, H, and I) among 10 clones (A-J). When we examined the expression levels of several kinds of T-cell surface markers in these 6 clones, the expression level of CD3 increased significantly. We selected the Jurkat-I culture, which expressed CD3 most strongly (Table 2) and applied to the following experiments. We also used cell cultures of the Jurkat clone E6-1 as positive control.
Statistical analysis

All results are presented as the mean ± SD. Statistical analyses were performed by using the t-test, and P values <0.05 were considered to be statistically significant.

Results

Expression levels of CD3 on the cell surface and cytoplastm

We measured the expression levels of CD3 (PE) in the cell surface and CD3 (FITC) in the cytoplastm in each of three cell lines by flow cytometry (FCM). The surface expression levels of CD3 in cultures of both Jurkat-I and Jurkat clone E6-1 cell lines were significantly higher than those in the Jurkat wild strain cell line (Fig. 1A). The expression levels of surface CD3 were 16.0% (MFI=1.16) in the Jurkat wild strain cells, 86.6% (MFI=7.48) in cells of the Jurkat-I clone, and 94.8% (MFI=49.3) in those of the Jurkat clone E6-1. On the other hand, the full expression of cytoplastic CD3 was observed equally in these three cell lines. The expression levels of CD3 in the cytoplastm were 97.7% (MFI=7.15) in Jurkat wild strain cells, 99.4% (MFI=32.1) in Jurkat-I cells, and 99.6% (MFI=22.2) in those of the Jurkat clone E6-1 (Fig. 1B).

Morphologies of Jurkat wild strain and Jurkat-I cells

Doubling times in both Jurkat wild strain and Jurkat-I cultures were about 24 h. Jurkat-I had cells that were larger in cellular size and with a lower nuclear-cytoplastic ratio, compared with cells of the Jurkat wild strain. Moreover many small vacuoles in the cytoplastm and cleaved nuclei were observed in Jurkat-I cells, showing a monocytoid appearance (Fig. 2), although neither surface CD14 nor cytoplastic butyrate esterase stain was negative (data not shown).

Expression levels of MYL9 of three cell lines and peripheral cells by RT-PCR

Naturally, Jurkat-I cells significantly expressed MYL9 mRNA, although cells from both the Jurkat wild strain and Jurkat clone E6-1 cultures did not express MYL9 mRNA (Fig. 3A). However, these three cell lines expressed MYL12A, MYL12B, and MYH9 mRNA (Fig. 3A). Expression of MYL9, MYL12B, and MYL12A mRNA were observed in normal peripheral pan T cells, platelets, B cells, and monocytes by RT-PCR (Fig. 3B).

The CD3 and MYL9 expression in Jurkat wild strain cells stimulated by PMA and A23187

The expression levels of CD3 (PE) increased to 76.5% (MFI=2.93) by treating with PMA (81 nM; 50 ng/mL) for 21 h (Fig. 4A), but MYL9 expression in RT-PCR was not observed (Fig.4A. inset). The expression levels of CD3 (PE) were 29.1% ± 6.65 (n=4) in Jurkat wild strain cells, 77.5% ± 6.27 in the
Fig. 1. The expression levels of surface CD3 and cytoplasm CD3. (A) The expression levels of CD3 (phycoerythrin [PE]) on the cell-membrane in cells from cultures of the Jurkat wild strain, Jurkat-I, and Jurkat clone E6-1. Flow cytometric analysis of the expression of both IgG (PE) (blue curve) and CD3(PE) (red curve) are shown for each of the Jurkat wild strain, Jurkat-I and Jurkat clone E6-1 cultures. (B) The expression levels of CD3 (fluorescein isothiocyanate [FITC]) in the cytoplasm by FCM (flow cytometry) after being permeated with IntraPrep™ (Beckman Coulter, Marseille Cedex 9, France). Flow cytometric analysis of the expression of both IgG (FITC) (blue curve) and CD3 (FITC) (red curve) are shown for cells of the Jurkat wild strain, Jurkat-I and Jurkat clone E6-1.

Fig. 2. May-Giemsa-stain image (×1,000). Jurkat wild strain cells (left) and Jurkat-I cells (right). The cellular size of Jurkat-I is slightly larger than that of the Jurkat wild strain cells and many small vacuoles are seen in the cytoplasm.
CD3 and MYL9 expressions in Jurkat cells

Jurkat wild strain cells with PMA (20 nM) for 21 h ($P<0.001$), 72.9% ± 8.87 in the Jurkat wild strain cells with PMA (81 nM) for 21 h ($P<0.001$) (Fig. 4B). The expression levels of CD3 were different between PE and FITC. The expression levels (%) of CD3 (FITC) also increased apparently by treating with PMA (20 nM) for 21 h; 1.44% ± 0.75 in the Jurkat wild strain cells and 44.3% ± 2.58 in the Jurkat wild strain cells with PMA (20 nM) ($P=0.001$, $n=3$) (Fig. 4C). A23187 (10 μM)-treatment for 30 min also showed an increase in expression levels (%) of CD3 (FITC), although to a lesser extent; 0.54% ± 0.10 ($n=4$) in the Jurkat wild strain cells and 12.1% ± 3.30 in the Jurkat wild strain cells with A23187 (10 μM) ($P=0.004$, $n=4$) (Fig. 4C).

Discussion

We previously identified three kinds of myosin regulatory light chains (MYL12A, MYL12B, and MYL9) from human blood cells (Watanabe et al., 2001; Higashihara et al., 2008). The mRNAs for both MYL12A and MYL12B are constitutively conserved in all lineage of leukemic cell lines determined thus far. However, MYL9 mRNA has only been detected in monocytic cell lines and erythroid cell lines (Higashihara et al., 2008). To confirm the function of MYL9 in blood cells, Jurkat-I, a new
clone was established by the transfection of MYL9 gene into cells of the Jurkat wild strain (acute T cell leukemia cell line) in which CD3 was expressed at a very low level on the cell surface, although with a high level of expression in the cytoplasm (Fig. 1A, 1B). In cells of the Jurkat wild strain, both MYL12A mRNA and MYL12B mRNA are fully expressed, but not MYL9 mRNA (Higashihara et al., 2008). Interestingly, Jurkat-I cells not only expressed monocytoid appearance (Fig. 2), but also had an elevated surface CD3 expression (from 16% to 86.6%) (Fig. 1A, Table 2). These results suggest that surface CD3 expression may have a causal relation to the myosin regulatory light chain 9 (MYL9)-dependent pathway in Jurkat cells.

Fig. 4. The expression of surface CD3 in cultures of the Jurkat wild strain treated with PMA or A23187. (A) Flow cytometric analysis of IgG (PE) (blue curve) and CD3 (PE) (red curve) after treatment of the Jurkat wild strain culture for 21 h with PMA (81 nM; 50 ng/mL). Inset shows the MYL9 and MYH9 mRNA expressions in RT-PCR. (B) The expression levels of CD3 (PE) in the Jurkat wild strain culture treated with PMA (20 nM and 81 nM) for 21 h. (C) The expression levels of CD3 (FITC) in the Jurkat wild strain culture treated with PMA (20 nM) for 21 h or A23187 (10 μM) for 30 min.

CD3 is the complex of polypeptides forming part of the TCR. In the human normal pan T cells, MYL9, MYL12A, and MYL12B have been expressed in mRNA levels (Fig. 3B). Moreover these three types of MLC mRNAs have been expressed in other mature cells (platelets, B cells, and monocytes) (Fig. 3B). In mature pan T cells, both CD3 and MYL9 are expressed. On the contrary, in cells of the T cell leukemia/lymphoma cell line such as the Jurkat wild strain, neither MYL9 mRNA nor surface CD3 was detected, although cytoplasmic CD3 was fully expressed. Thus it is suggested that in the Jurkat wild strain, the transport of CD3 from cytoplasm to surface membrane is interrupted. The heterogeneity of the CD3 surface level is a common feature of many pathologies including T cell leukemia/lymphoma cells (Suzushima et al., 1991; Yokote et al., 2005). Jurkat cell lines with different CD3
expression have been cloned by limiting dilutions (Jóźwik et al., 2004). The surface CD3 level of the Jurkat wild strain cells we used was very low, but was very high in cells of the Jurkat clone E6-1 (Fig. 1A). However, MYL9 mRNA was not detected in cells of the Jurkat clone E6-1 (Fig. 3A), which had high levels of surface CD3 (Fig. 1A).

The central event in T cell activation is the interaction of the T cell receptor (TCR) with the antigenic peptide presented by the major histocompatibility complex (MHC) of the antigen-presenting cell (APC). The linker activator for T cells (LAT) is an integral membrane adaptor protein. LAT stimulates one critical protein phospholipase C (PLC) that is responsible for the production of the second messengers Diacylglycerol (DAG) and inositol triphosphate (IP3) by cleaving phosphatidylinositol-4,5- bisphosphate (PIP2) at the plasma membrane. DAG activates a number of proteins, such as PKC, whereas IP3 binds to IP3 receptor on the surface of the endoplasmic reticulum and releases Ca2+ (Abraham et al., 2004).

PKC-θ is a serine/threonine kinase belonging to the calcium-independent novel PKC subfamily, and is expressed in T cells. The signals delivered from T cell receptors (TCRs) and CD28 co-stimulatory molecules trigger PKC-θ catalytic activation and membrane translocation to the immunological synapse, leading to activation of nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1), and nuclear factor of activated T cell (NFAT). Phosphorylation of PKC-θ at multiple Ser/Thr/Tyr residues is induced in T cells during TCR signaling (Wang et al., 2012).

The increased Ca2+ levels then activate the protein phosphatase calcineurin (Klee et al., 1979) by disrupting the inhibitory effects of calmodulin. Calcineurin activation leads to the dephosphorylation of NFAT, allowing it to enter the nucleus, where it co-operates with other transcription factors to bind promoters (Burbach et al., 2007).

In the present study, we examined the role of two main T cell signal transduction pathways in controlling the surface level of CD3 antigen, one based on protein kinase C (PKC) activity and the other dependent on calcineurin. The cells were stimulated with PMA or Ca2+- ionophore A23187 (one of the Ca2+-mobilizing compounds), acting directly on PKC or calcineurin, respectively. The effect of PMA on surface CD3 expression was prominent compared with that of A23187 (Fig. 4B, 4C), suggesting the important involvement of PKC in the regulation of surface CD3 expression. PKC can phosphorylate the regulatory light chain (RLC) on Ser1, Ser2 and Thr9 to inhibit non-muscle myosin II (NM II) (Nishikawa et al., 1984; Vicente-Manzanares et al., 2009). Jóźwik et al. (2004) reported that differences in the expression of CD3 in Jurkat clone E6.1 cells are in parallel with the activity of PKC. Therefore, we speculate that the PKC-dependent pathway is the important way of controlling surface CD3 expression, even though MYL9 was not expressed (Fig. 4A, inset). CD3 antigen is a crucial molecule in T cell signal transduction. Although its expression on the cell surface is constitutive, a dynamic regulation of the TCR-CD3 level is probably the most important mechanism allowing T cells to calibrate their response to different levels of stimuli.

Recent study indicated that knockdown of MYL12A/12B genes in NIH 3T3 fibroblasts resulted in striking changes in cell morphology and dynamics and the levels of MYH9 were reduced significantly in the MYL12A- and MYL12B- knockdown fibroblasts (Park et al., 2011). In their study, MYL9 could not be knocked down with real-time PCR. We attempted to knockdown the gene of MYL9 in U937, which was the original cell line to clone the MLC-2C (MYL9) (Higashihara et al., 2008), but the results were unsuccessful. In the future, we need to knockdown the MYL9 gene of T cells and leukemic cell
lines that express MYL9 to further investigate changes in cell morphology and dynamics.

The importance of myosin motors in processes related to T cell activation is further highlighted by the phosphorylation and subsequent activation of the myosin light chain after TCR engagement (Rey et al., 2007). During T cell activation, the engagement of co-stimulatory molecules is often crucial to the development of an effective immune response, and depends on myosin motor proteins (Wülfing et al., 1998). However, the precise role of myosins in T cell activation also warrants further exploration.

In summary, Jurkat-I cell cultures established by the transfection with the MYL9 gene, expressed CD3 on the cell surface. PMA increased the surface CD3 levels in Jurkat wild strain cells without MYL9 gene expression. These results suggest that surface CD3 expression proceeds through both MYL9-dependent and MYL9-independent pathways (i.e. PKC-dependent pathway) in Jurkat cells.

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