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REVIEW ARTICLE

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T-cell activation via CD26 and caveolin-1 in rheumatoid synovium

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Abstract CD26 is a T-cell costimulatory molecule with dipeptidyl peptidase IV (DPPIV) activity in its extracellular region. We previously reported that recombinant soluble CD26 enhances peripheral blood T-cell proliferation induced by the recall antigen tetanus toxoid (TT). Recently, we demonstrated that CD26 binds caveolin-1 on antigen-presenting cell (APC), and that residues 201–211 of CD26 along with the serine catalytic site at residue 630, which constitute a pocket structure of CD26/DPPIV, contribute to binding to caveolin-1 scaffolding domain. In addition, following CD26–caveolin-1 interaction on TT-loaded monocytes, caveolin-1 is phosphorylated, with linkage to NF-κB activation, followed by upregulation of CD86. Finally, reduced caveolin-1 expression on APC inhibits CD26-mediated CD86 upregulation and abrogates CD26 effect on TT-induced T-cell proliferation, and immunohistochemical studies revealed an infiltration of CD26+ T cells in the sublining region of rheumatoid synovium and high expression of caveolin-1 in the increased vasculature and synoviocytes of the rheumatoid synovium. Taken together, these results strongly suggest that CD26–cavolin-1 interaction plays a role in the upregulation of CD86 on TT-loaded APC and subsequent engagement with CD28 on T cells, leading to antigen-specific T-cell activation such as the T-cell-mediated antigen-specific response in rheumatoid arthritis.

Key words Caveolin-1 · CD26 · Memory T cell · Rheumatoid arthritis (RA) · Synovial cell

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the progressive destruction of cartilage and bone in the synovial joints, which is associated with proliferation of synovial cells and infiltration of activated memory T cells, antigen-presenting cells (APCs) and plasma cells.[1] Proposed etiologies for RA include genetic predisposition, dysregulation of self-tolerance, immune dysregulation triggered by environmental agents, and subsequent transformation of synovial cells.[1–3] Macrophages and/or T cells are important mediators of RA pathogenesis, with cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) being proven therapeutic targets. In fact, antagonists against such cytokines have been used recently as effective RA therapy, decreasing joint damage and slowing radiographic progression of disease in patients of RA with inadequate response to methotrexate.[4–7] However, as many patients do not experience effective relief even with the use of these newer biological agents, additional novel therapeutic approaches are still needed.[8–10]

Major-histocompatibility-complex (MHC) class II phenotype such as HLA-DR1, DR-4 and DR-14 confers susceptibility to RA.[11–14] MHC class II molecules present antigens to CD4+ T cells, suggesting an important role for T cells in the pathogenesis of RA. Moreover, the rheumatoid synovium contains activated T cells, providing further rationale for the proposal that T cells have an important role in RA.[15,16] Antigen-presenting cells such as monocytes, macrophages, and dendritic cells are also present in the rheumatoid synovium,[1] being activated and expressing both MHC class II and costimulatory molecules such as CD86 and CD80. These findings strongly suggest that the interaction between synovial T cells and APCs have a direct role in the progression of synovitis.[2] Moreover, careful analysis of infiltrating synovial T cells has revealed a bias towards the T_H1 phenotype.[17,18] In particular, patients with autoimmune diseases such as multiple sclerosis, Graves' disease, and RA have been found to have increased numbers of CD4+

CD26+ T cells in inflamed tissues as well as in their peripheral blood,[19-22] with enhancement of CD26 expression in these autoimmune diseases correlating with disease severity.[19,20,23] In addition, we previously demonstrated that T cells migrating through endothelial cell monolayers in vitro express high levels of CD26.[24] These findings imply that CD26+ T cells play an important role in the inflammation process and subsequent tissue damage in such diseases.

It is well established that T cells require at least two signals to be fully activated.[25] The first signal is antigen-specific and is delivered by engagement of the T-cell receptor (TCR) complex with an MHC-peptide complex on APC. The second signal is exerted by the binding of a costimulatory receptor on T cells to a ligand on the APCs. A key costimulatory signal is provided by the interaction of CD28 on T cells with CD86 or CD80 on APCs. We showed previously that CD26 on T cells have a very strong costimulatory effect on CD4+ T-cell activation in response to memory antigen such as tetanus toxoid (TT).[26-29] However, the molecular mechanism involved in the process of antigen-specific T-cell activation via CD26 has not been clearly elucidated. We recently demonstrated that caveolin-1 on antigen-loaded monocytes is a binding partner of CD26 and that signaling downstream of caveolin-1 in APC is triggered by stimulation with exogenous CD26.[30,31] Therefore, T-cell costimulation via CD26 as well as CD28 may have an important role in the pathophysiology of inflammatory diseases such as RA. In this review, we discuss various aspects of CD26 involvement in immune regulation and immune-mediated disorders such as RA, with a particular focus on the role of caveolin-1 as its key binding partner.

Structure and function of CD26

CD26 is a 110 kDa cell-surface glycoprotein that belongs to the serine protease family, and human CD26 is expressed on a variety of tissues including T lymphocytes, endothelial and epithelial cells. As shown in Fig. 1A, human CD26 is composed of 766 amino acids, including a short cytoplasmic domain of 6 amino acids, a transmembrane region of 24 amino acids, and an extracellular domain with dipeptidyl peptidase activity which selectively removes the N-terminal dipeptide from peptides with proline or alanine at the penultimate position (dipeptidyl peptidase IV, DPPIV).[32] The amino acid sequence of human CD26 illustrates approximately 85% homology with the rat DPPIV enzyme and the mouse thymocyte activation molecule (THAM), the mouse homologue of human CD26.[33] CD26 knockout (CD26-KO) mice with C57BL/6 background display an apparently normal phenotype.[34,35] However, the percentage of CD4+ T cells is lower in the spleen lymphocyte population in the CD26-KO mice than in CD26-positive wild-type mice. After immunization of mice with PWM in vivo, serum levels of total IgG, IgG_1, IgG_{2a} and IgE were markedly decreased in CD26-KO mice than those in wild-type mice. Moreover, IL-4 and IL-2 level in sera of CD26-KO mice were decreased and production of interferon-gamma (IFN-γ) was delayed in response to PWM immunization. These results indicate that CD26 helps to regulate the development, maturation and migration of CD4+ T lymphocytes, cytokine secretion, T cell-dependent antibody production, and immunoglobulin isotype switching of B cells.[34]

In contrast to the function of murine CD26, human CD26+ T cells exert diverse effects.[28,36,37] CD26 is a membrane-associated ectopeptidase with DPPIV activity, and possible substrates of CD26/DPPIV include several critical cytokines and chemokines. Activity of RANTES (regulated on activation, normal T-cell expressed and secreted; CCL5) is altered by the enzymatic cleavage of DPPIV, as CD26/DPPIV-processed RANTES affects important activities such as those implicated in monocyte chemotaxis and HIV-1 infection.[38,39] Other important chemokines that appear to be substrates of DPPIV enzymatic activity include eotaxin (CCL11), macrophage-derived chemokine (MDC) (CCL22), interferon inducible chemokines (CXCL10), and other chemokines involved in the inhibition of HIV infection.[39] In addition, recent work showed that CD26 plays an important role in the mobilization of hematopoietic stem cell (HSC) and hematopoietic progenitor cells (HPC) induced by granulocyte colony-stimulating factor (G-CSF).[40] One of the substrates of CD26/DPPIV is CXCL12 (SDF-1α, stromal cell-derived factor 1 alpha), an important chemokine that serves as a chemoattractant for HSC/HPC.[41,42] It has been shown that CXCL12 can be selectively truncated in vitro by CD26/DPPIV, and the truncated molecule lacks the ability to induce migration of hematopoietic cells isolated from mouse bone marrow. Furthermore, treatment of mice with CD26/DPPIV inhibitors during the process of G-CSF mobilization results in a significant reduction in the number of mobilized HPC.[40,41] Other exciting development regarding DPPIV involves its role in glucose metabolism, since inhibition of endogenous glucagon-like peptide 1 (GLP-1) degradation by reducing DPPIV activity is an alternative strategy for improving the incretin action of GLP-1 in vivo and regulating glucose levels.[43] Selective small molecule inhibitors of DPPIV are currently being investigated in clinical trials for the treatment of impaired glucose tolerance and type 2 diabetes.[44]

Besides its ability to regulate the effect of biological factors through DPPIV enzyme activity, CD26 has an essential role in human T-cell physiology, especially in response to memory antigens (Fig. 1B).[28] Originally characterized as a T-cell differentiation antigen, CD26 is preferentially expressed on a specific population of T lymphocytes, the subset of CD4+ CD45RO+ memory T cells, and is upregulated following T-cell activation.[29] Besides being a marker of T-cell activation, CD26 is also associated with T-cell signal transduction processes as a costimulatory molecule.[27,37,45,46] In addition, CD26 serves as a functional collagen receptor with a role in T-cell activation, as well as having a potential role in thymic ontogeny (Fig. 2).[26,46,47] The enzymatic activity of CD26 appears to be very important in enhancing cellular responses to external stimuli. For example, Jurkat cells transfected with wild type CD26 consis-

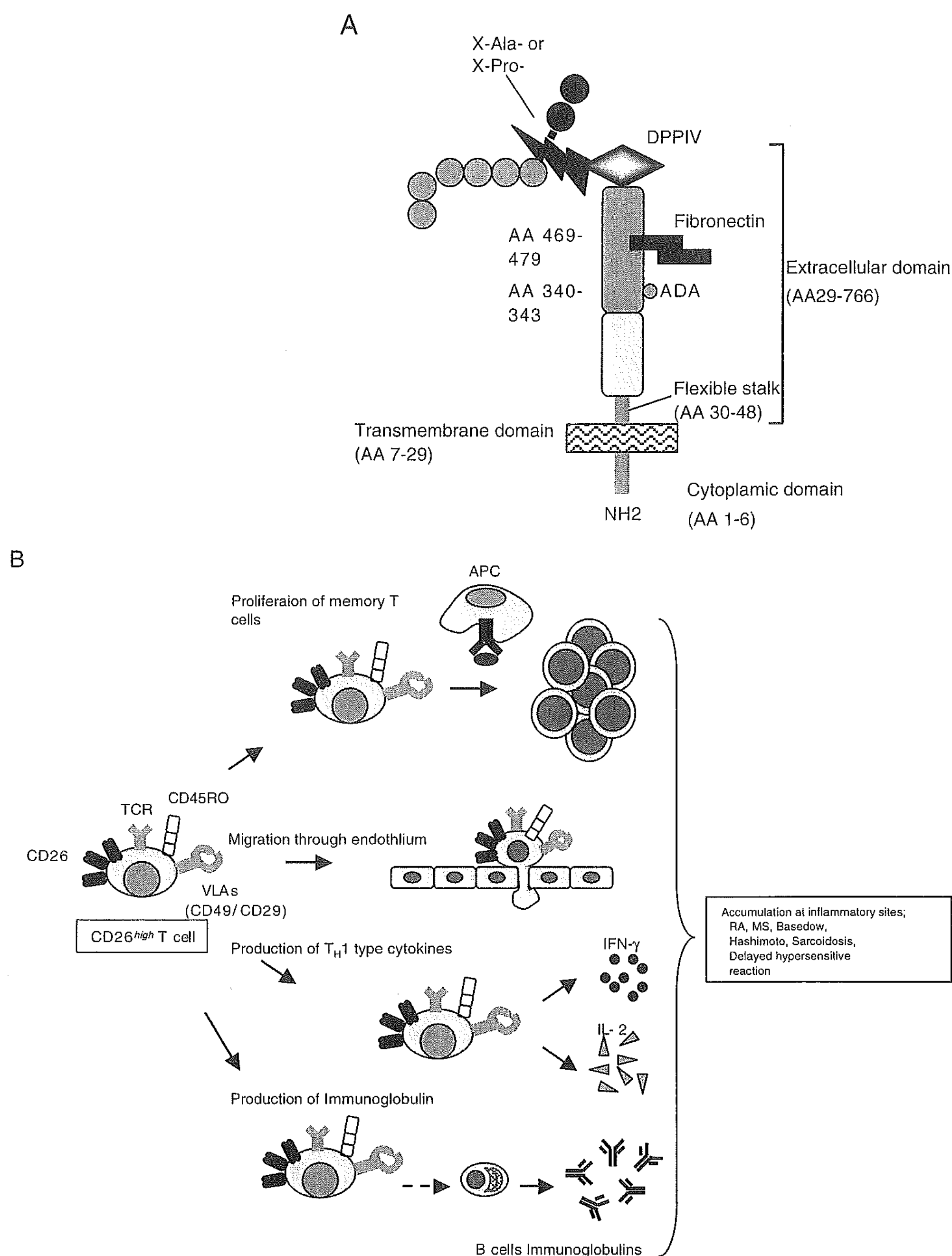


Fig. 1. **A** Schematic diagram of human CD26 structure. Adenosine deaminase (*ADA*) binding site at residues 340–343, fibronectin binding site at residue 469–479, and dipeptidyl peptidase IV (*DPPIV*) enzyme activity at Ser630. *X-Ala-* or *X-Pro-* denotes peptides containing any amino acid at N-terminal position with alanine or proline at the penultimate position. **B** Cellular function of CD26^{high} T cell. See text for details. *APC*, antigen-presenting cell; *TCR*, T-cell receptor; *IFN*, interferon; *IL*, interleukin

tently demonstrate greater activation than parental CD26 negative Jurkat or cells transfected with CD26 mutated at the DPPIV enzymatic site.[48] Furthermore, CD26 interacts with several molecules playing important roles in T-cell function. CD26 physically binds with adenosine deaminase (ADA), an enzyme that plays a key role in the development and function of lymphoid tissues.[49–51] Adenosine deaminase is essential for purine metabolism, with the loss of ADA leading to a clinical syndrome characterized by severe immunodeficiency.[52] When the ADA inhibitor pentostatin was used in the treatment of recurrent T-cell lymphomas, a significant reduction in circulating CD26+ T cells was observed in treated patients.[53] This finding is consistent with the fact that there is a physical association between CD26 and ADA on the surface of T lymphocytes. CD26 also interacts with CD45RO, a tyrosine phosphatase with a critical role in T-cell signal transduction, at lipid rafts in peripheral blood T lymphocytes to modify cellular signaling events (Fig. 2).[54,55] Interestingly, CD26 is associated with CD45 RA outside of lipid rafts in cord blood T cells, and the strong physical linkage of CD26 and CD45 RA may be responsible for the attenuation of cord blood T-cell activation signaling through CD26, which may in turn result in immature immune response and the relatively low incidence of severe graft-versus-host disease (GVHD) in cord blood transplantation (Fig. 2).[56]

Since the 1970s, DPPIV-like activity has been reported in human serum. After identification of the ADA-binding

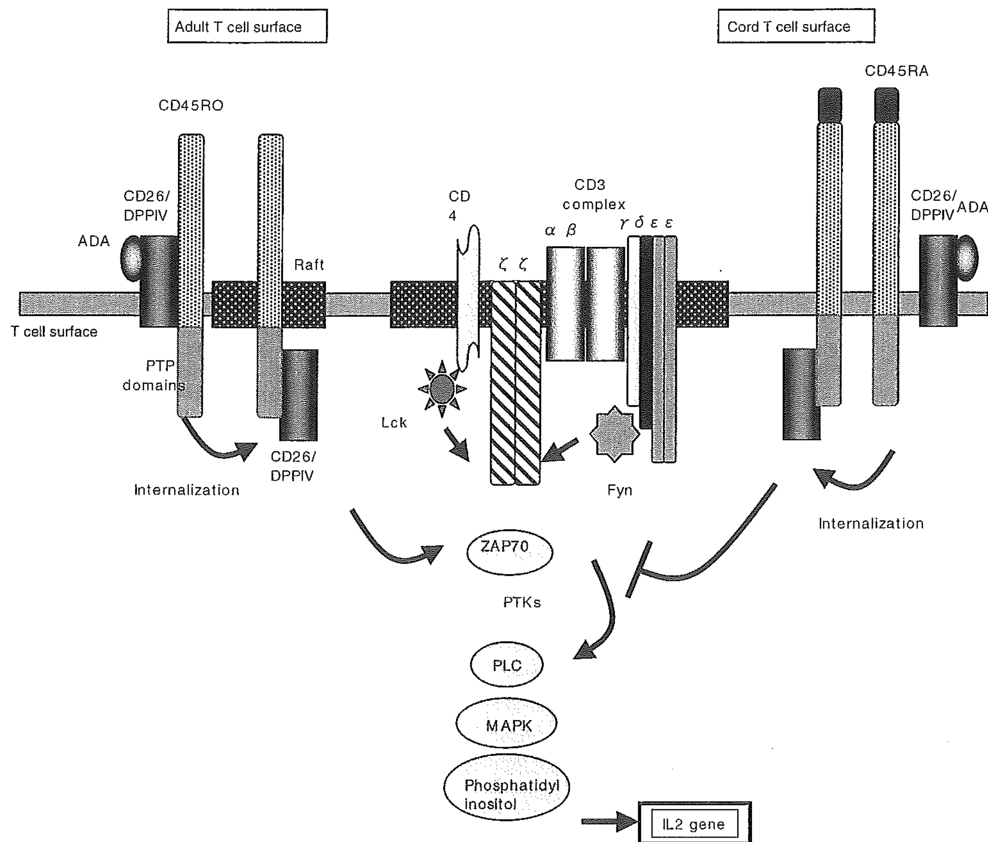


Fig. 2. Schematic diagram of CD26-associated molecules in T-cell receptor-mediated activation of human adult peripheral blood T cell and cord blood T cell

protein of plasma as CD26, soluble form of CD26 protein was characterized in the serum and seminal fluids.[57,58] In the previous report, we have shown that exogenous recombinant soluble CD26 (rsCD26) enhances the proliferative response of peripheral blood lymphocytes (PBLs) to stimulation with the soluble antigen tetanus toxoid (TT).[59] More recently, we demonstrated that the target cells of rsCD26 are the CD14+ monocytes in the peripheral blood, and that rsCD26 upregulates CD86 expression, but not CD80 or HLA-DR antigen levels on monocytes.[30] Significantly, mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF-IIR) was identified as a platform molecule for CD26 interaction with APC.[30] However, while both DPPIV-positive and DPPIV-negative rsCD26 are taken up by monocytes via M6P/IGF-IIR, only DPPIV-positive rsCD26 molecules affect CD86 upregulation on monocytes, thus suggesting that additional key factors may interact with CD26 in this process. We subsequently identified caveolin-1 on APC as a binding protein for CD26, and demonstrated that CD26 stimulation upregulates surface expression of CD86 on APC by means of caveolin-1 and enhances TT-mediated T-cell proliferation.[31] In the next section of this review, we will focus on caveolin-1 as the binding protein of CD26 in the context of antigen-driven T-cell activation.

Structure and function of caveolin-1

Caveolin-1 was the first family member discovered, and demonstrated as a structural component and marker for caveolae and trans-Golgi derived transport vesicles.[60,61] Caveolae were described as structures resembling "little caves" due to their appearance as 50- to 100-nm vesicular invaginations of the plasma membrane.[62] Caveolin-1 is expressed in a wide variety of cell types, especially terminally differentiated cells such as endothelial cells, adipocytes, alveolar type I pneumocytes, macrophages, synoviocytes, and smooth muscle cells. Presently, caveolin-related proteins have been identified as caveolin-1, -2, and -3, all of which serve as protein markers for caveolae.[63] The majority of caveolae in cells and tissues require only caveolin-1 expression for their proper formation, whereas caveolin-2 is not absolutely required, although the expression of caveolin-2 is tightly associated with the expression of caveolin-1.[64,65] On the other hand, caveolin-3 is found in skeletal muscle tissue and cardiac myocytes.[66] The three human genes encoding members of the caveolin family share significant homology. The caveolin-2 protein is approximately 38% identical and 58% similar to caveolin-1, while caveolin-3 is more closely related to caveolin-1, with 65% identity and 85% similarity.[63] All three caveolins possess an invariant "FEDVIAEP" stretch within their hydrophilic N-terminal domains which

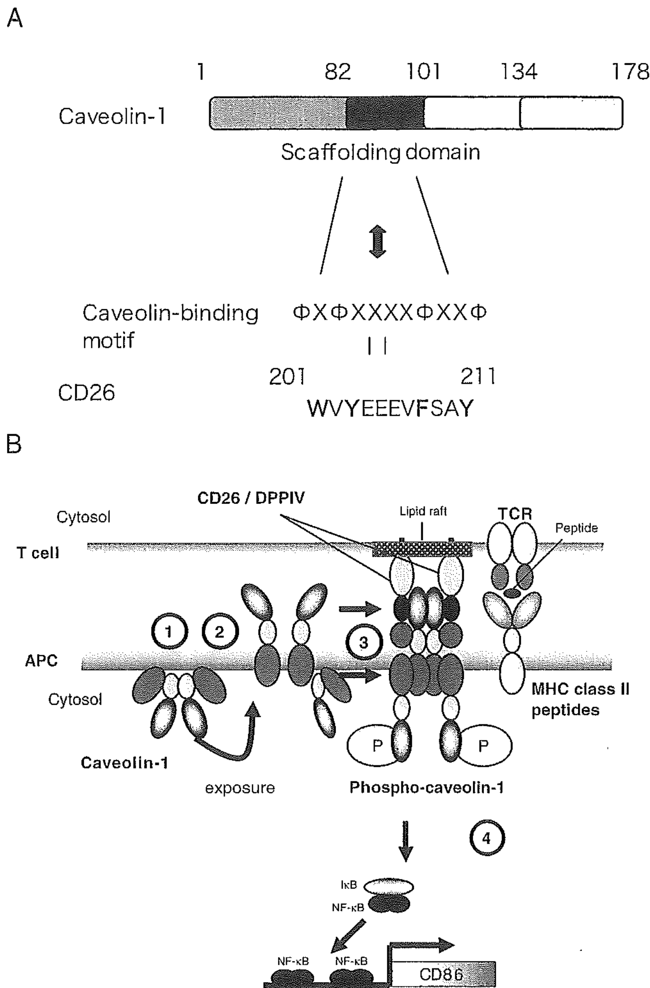


Fig. 3. **A** Schematic representation of human caveolin-1. Residues 1–81 comprise the N-terminal region (NT; *striped rectangle*), residues 82–101 comprise the scaffolding domain (SCD; *black rectangle*), residues 102–134 comprise the transmembrane region (memb; *open rectangle*), and residues 135–178 comprise the C-terminal region (*dotted rectangle*). CD26 contains a caveolin-binding motif (*ΦXΦXXXXΦXXΦ*; Φ and *X* depict aromatic residue and any amino acid, respectively), specifically WVYEEEVFSAY in CD26. **B** Model for CD26–caveolin-1 interaction leading to upregulation of CD86. *(1)* Caveolin-1 in monocytes (antigen-presenting cells; *APC*) resides at the inner membrane. *(2)* After uptake of tetanus toxoid into monocytes via caveolae, part of the population of caveolin-1 is exposed on the outer cell surface of tetanus toxoid (TT)-loaded monocytes. *(3)* Migration of CD26+ antigen-specific memory T cells to areas of antigen-loaded APCs results in contact with TT antigen-presenting APC, leading to the association of CD26 and caveolin-1. Aggregation of caveolin-1 in the contact area occurs, presumably by homo-oligomerization (via its residues 61–101), followed by its phosphorylation. *(4)* Phosphorylated caveolin-1 (*phospho-caveolin-1*) transduces signaling leading to activation of NF-κB, resulting in CD86 upregulation. *DPPIV*, dipeptidyl peptidase IV; *TCR*, T-cell receptor; *MHC*, major histocompatibility complex

are named the "caveolin signature motif."[67] Caveolin-1 is composed of 178 amino acid residues (Fig. 3A), and predominantly localized at the plasma membrane, demonstrating a punctuate staining patterns, and in Golgi-derived vesicles.[60] Two isoforms of caveolin-1 (caveolin-1α and β) have been identified, with the β-isoform composed of 31 residue truncated N-terminus of caveolin-1α isoform.[68] Caveolin-1 is composed of the N-terminal hydrophilic domain (residues 1–101), the oligomerization domain (residues 61–101), the scaffolding domain (SCD) (residues 82–101), the membrane spanning domain (residues 102–134), and the C-terminal lipid raft-anchoring domain (residues 135–178).[63] As in trans-Golgi transport, caveolin-1 plays an important role in signal transduction via its SCD, which compartmentalizes a multitude of signaling molecules.[63,69] These include G proteins, epidermal growth factor receptor, insulin receptor, endothelial nitric oxide synthase (eNOS), nonreceptor tyrosine kinase (Src, Fyn, Yes), flotillins, Ser/Thr kinases (PKA, Raf, MAPK, PI3K, Grb2), and catenins.[63,69] Other cellular functions of caveolin-1 are related to the lipid metabolism, especially to cholesterol scavenging in macrophages.[70] However, it is unknown whether caveolin-1 also plays a role in signal transduction in APCs. Although CD26 was present in caveolae of fibroblast-like synoviocytes,[71] direct CD26–cavolin-1 interaction and associated signaling events have not been demonstrated in immune cells. Interestingly, caveolin-1 knockout mice show defects in the angiogenic response to exogenous stimuli, such as Matrigel plugs containing angiogenic growth factors (bFGF) or tumors.[72] In this context, angiogenic vessels density and penetration was significantly reduced in caveolin-1 null mice. Moreover, electron microscopic examination revealed incomplete de novo capillary formation in tumors implanted within caveolin-1 null mice. Thus, it appears that caveolin-1 null mice have a defect in endothelial cell differentiation. This is consistent with in vitro observations demonstrating that overexpression of caveolin-1 enhances endothelial capillary-tube formation, while downregulation of caveolin-1 using an anti-sense approach blocks endothelial tube formation.[73] With regards to inflammation and caveolin-1, a series of elegant experiments showed that caveolin-1 has a role in inflammation with association of eNOS.[74] Using a cell permeable peptides link to the caveolin-1 scaffolding domain in aortic explants, the potent eNOS inhibiting activity of caveolin-1 was demonstrated. In vivo delivery of this peptide resulted in significant decreases in acute inflammation and edema resulting from vascular permeability. Taken together, these findings demonstrate an important relationship between caveolin-1 and vascularization, with implication for capillary formation in inflammatory processes.

Caveolin-1: CD26 binding protein in APC

Since CD26 on human T cells was identified as an activation antigen and costimulatory molecule of the TCR complex, several binding proteins to CD26 have been described. As described above, multiple chemokines interact with CD26/DPPIV as its substrates, and other proteins such as ADA, fibronectin, thromboxane A2 receptor, and CXCR4 are shown to be associated with CD26.[49,75–78] However, the precise mechanism involved in T-cell activation in response to memory antigen such as TT remains to be clearly characterized. Recently, we demonstrated that CD26 binds to

caveolin-1 on APC, and that residues 201–211 of CD26 along with the serine catalytic site at residue 630, which constitute a pocket structure of CD26/DPPIV, contribute to binding to the caveolin-1 scaffolding domain (Fig. 3A).[31] This region in CD26 contains a caveolin-binding domain (CBD) ($\Phi X \Phi XXXX \Phi XX \Phi$; Φ and X depict aromatic residue and any amino acid, respectively), specifically WVYEEEVFSAY in CD26.[48,69] These observations strongly support the notion that DPPIV enzyme activity is necessary to exert TCR-costimulatory activation via CD26.[48] In addition, following CD26–cavolin-1 interaction on TT-loaded monocytes, caveolin-1 is phosphorylated, with linkage to NF-κB activation, followed by upregulation of CD86. Finally, reduced caveolin-1 expression on monocytes inhibits CD26-mediated CD86 upregulation and abrogates CD26 effect on TT-induced T-cell proliferation (Fig. 3B). Taken together, these results strongly suggest that CD26–cavolin-1 interaction plays a role in the upregulation of CD86 on TT-loaded monocytes and subsequent engagement with CD28 on T cells, leading to antigen-specific T-cell activation.

Caveolin-1 has been reported to be an integral membrane protein with a cytoplasmic N-terminal domain and a cytoplasmic C-terminal domain.[63] Our data showed that the N-terminal domain of caveolin-1 was expressed on the cell surface of monocytes 12–24 h after tetanus toxoid was loaded (Fig. 4A). Since tetanus toxoid was trafficked in cells through caveolae,[79,80] caveolin-1 may be transported along with the peptide-MHC complex in APC, and is then expressed on cell surface by the antigen-processing machinery for T-cell contact.[80–82] The data shown in Fig. 4B indicated that CD26 on activated memory T cells directly faces caveolin-1 on TT-loaded monocytes in the contact area, which is the immunological synapse for T cell-APC interaction. It is conceivable that the interaction of CD26 with caveolin-1 on antigen-loaded monocytes results in CD86 upregulation, therefore enhancing the subsequent interaction of CD86 and CD28 on T cells to induce antigen-specific T-cell proliferation and activation.

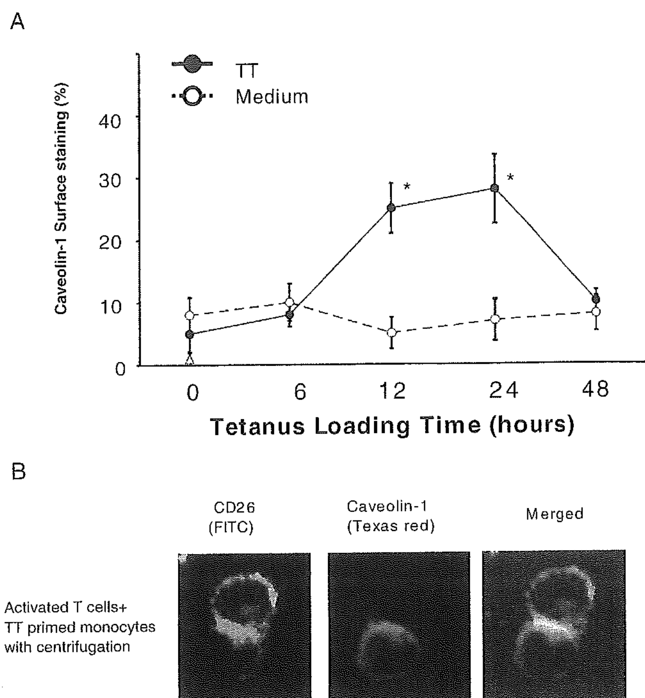


Fig. 4A,B. Immunocytochemical analysis of caveolin-1 and CD26 interaction. **A** Caveolin-1 in monocytes was exposed to cell surface after tetanus toxoid (*TT*) treatment, and interacted with CD26 on activated T cells. After purified monocytes were incubated with (*solid circle*) or without (*open circle*) TT, cell surface caveolin-1 was stained with anti-caveolin-1 antibody detecting the N terminal region, and analyzed for % positive cells by flow cytometry. Data of % positive cells represent mean ± SE from five independent experiments. *Asterisks* indicate points of significant increase. **B** To form cell–cell conjugation, activated T cells and TT-loaded monocytes were mixed, followed by centrifugation. Conjugates were fixed without permeabilization, and stained with anti-CD26 (fluorescein isothiocyanate) and anti-caveolin-1 (Texas red) antibodies. *Bars* 10 μm

CD26 and caveolin-1 in synovitis

Rheumatoid arthritis is a classical example of an immune-mediated disease with chronically smoldering injury of the synovial joints resulting from infiltration of inflammatory cells, and synovitis of diarthrodial joints is its most visible manifestation. Although the observed architectures of rheumatoid synovitis vary in different individuals with RA as well as at various disease stages, the most frequent type of rheumatoid synovitis is a diffuse inflammatory infiltrate in which T cells, B cells, and macrophages are scattered around increased vasculature and synoviocytes. Meanwhile, in the remaining 40–50% of patients with RA, infiltrating inflammatory cells organize themselves into follicular structures.[1] It is known that the inflammatory activation events in rheumatoid synovitis are dependent upon cell–cell contact among T cells, fibroblast-like synoviocytes, APCs, and regional tissues such as type II collagen and proteoglycan.[83] Moreover, previous reports showed that CD26+ T cells exhibit strong migratory ability through endothelial cells, and are present at high levels in the rheumatoid synovium and the synovial fluids.[20,22,23] These findings suggest that T cells with high levels of CD26 antigen may preferentially migrate into the rheumatoid synovium to induce inflammation and tissue destruction. To test this hypothesis, we examined the expression of CD26 and caveolin-1 in the rheumatoid synovium through immunohistochemistry. As shown in Fig. 5A, CD26+ lymphoid cells are clearly observed in diffuse synovitis. In follicular synovitis examined with the sequential sections, CD26+ lymphoid cells are infiltrated in the sublining area of caveolin-1-positive synovial cells (arrow in panel b of Fig. 5B), and are adjacent to caveolin-1-positive inflammatory cells (arrowheads in panel c of Fig. 5B). Moreover, the intimal lining layer is hyperplastic with multiple layers, and the synoviocytes in these layers highly express caveolin-1 (arrow in Fig. 5C). In addition, CD86 and caveolin-1 are coexpressed in the intimal lining synoviocytes and the sublining fibroblast-like synovial cells (black arrowhead in Fig. 5C). Furthermore, increased vas-

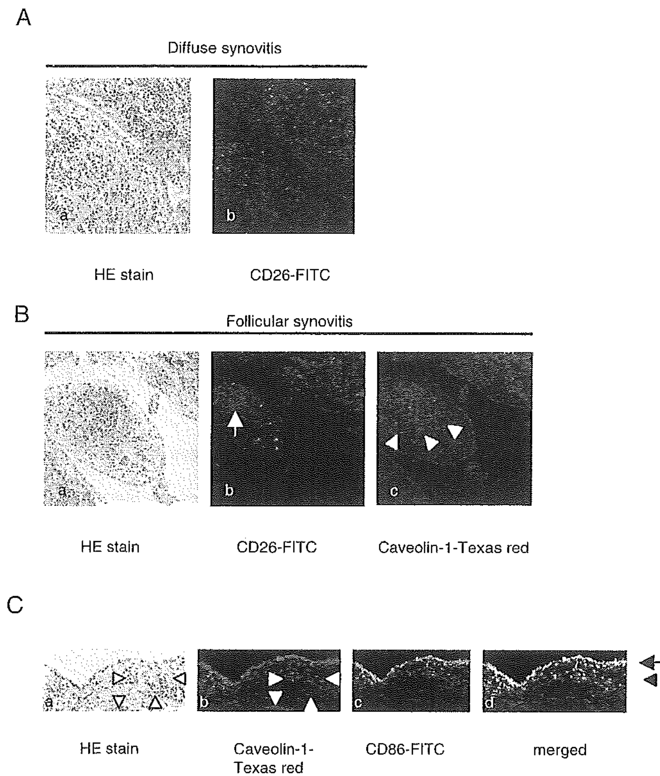
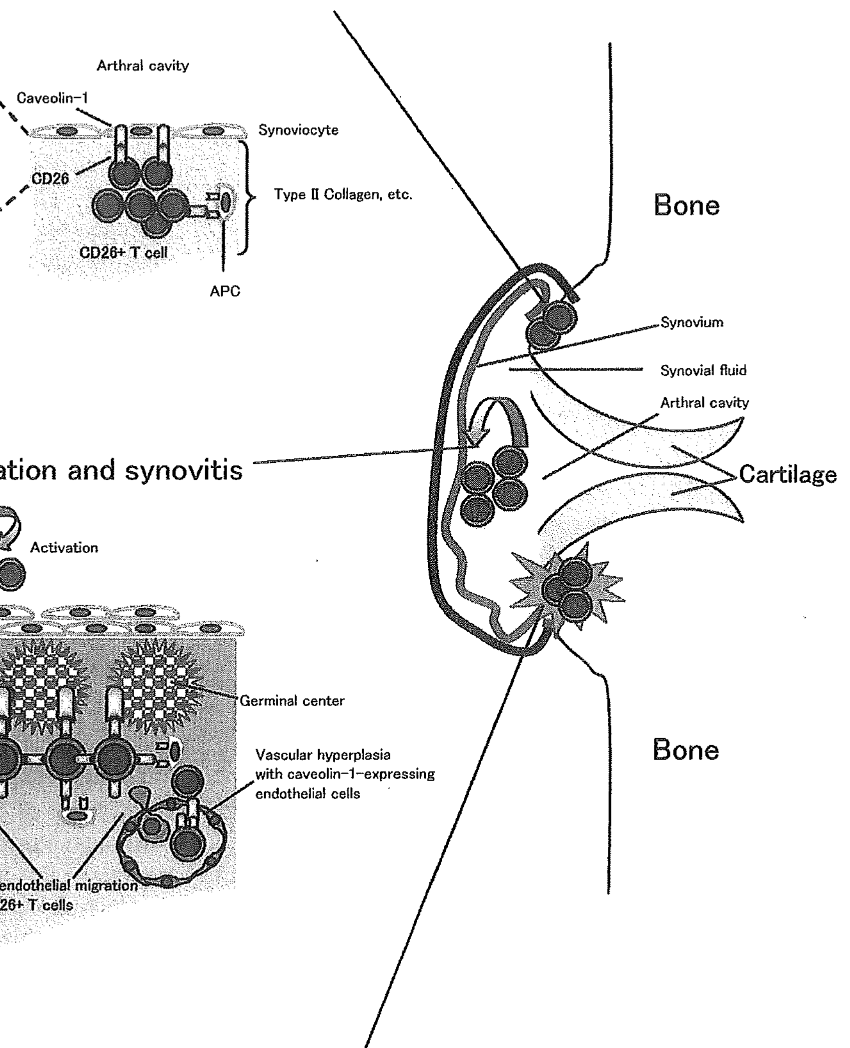


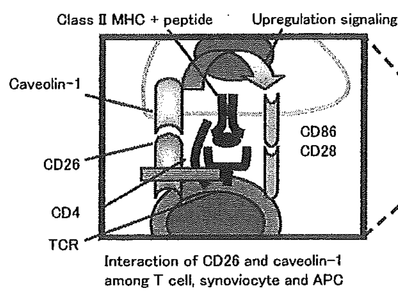
Fig. 5A–C. Architecture and immunohistochemistry of rheumatoid synovitis. **A** *Panel a* shows H&E-stained histology of diffuse synovitis with inflammatory cells intermingled with fibroblast-like synoviocytes (×100). *Panel b* shows immunohistochemistry of the sequential section of panel a, which was stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD26 antibody (×100). **B** *Panel a* shows H&E-stained histology of follicular synovitis with germinal center formation (×100). *Panel b* shows immunohistochemistry of the sequential section of panel a, which was stained with FITC-conjugated anti-CD26 antibody. This reveals that CD26-positive lymphoid cells are scattered around the follicles (*arrow*) (×100). *Panel c* shows immunohistochemistry of the sequential section of panel b, which was stained with anti-caveolin-1 (Texas red). This reveals that the intimal lining synoviocytes and the sublining fibroblast-like synoviocytes adjacent to CD26+ lymphoid cells (arrow head) express caveolin-1 (×100). **C** *Panel a* shows synovial histology of rheumatoid arthritis with H&E staining (×100). *Panels b and c* show immunohistochemistry of the sequential section of panel a which was stained with caveolin-1 (Texas red) and CD86 (FITC), simultaneously. *Panel d* shows the merged view of panels b and c. *Arrow* shows the intimal lining synoviocytes, and *black arrowhead* shows the sublining fibroblast-like synoviocytes. *White arrowheads* show the increased vascularization in synovitis

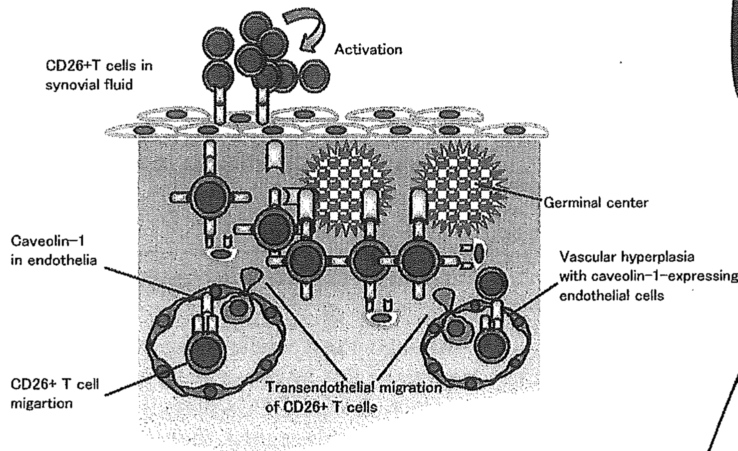
cularization is seen in synovitis, and caveolin-1 is preferentially expressed in the luminar surface of endothelial cells in the rheumatoid synovium (white arrowheads in Fig. 5C). Taken together, we propose a model to describe the molecular events in monocytes leading to activation that are triggered by CD26–cavolin-1 interaction in rheumatoid synovium (Fig. 6). The initial step of inflammation in the synovium proceeds from activation of CD26+ T cells by APC and/or rheumatoid synoviocytes via presentation of a yet-to-be-identified antigen, concomitant with costimulation via such pairing as CD28–B7 and CD26–cavolin-1 (phase 1 in Fig. 6). With regard to the interaction between T cell–APC and the resultant immune response, entry of antigens via caveolae into APC leads to presentation of antigen peptides on MHC class II molecules and exposure of caveolin-1 (inside box in phase 1 of Fig. 6). APC thus induces the activation of memory T cells through the TCR and costimulatory molecules such as CD86/CD80–CD28, leading to the formation of mature immunological synapses. Following the association between caveolin-1 on APC and CD26 on memory T cells, CD86 is upregulated on APC surface, and memory T cells are subsequently activated via the costimulatory effect of CD26 on TCR activation. By enhancing TCR activation via CD26–cavolin-1 interaction, prolongation of the immunological synapse may be maintained. CD86 upregulation therefore results in potent T cell–APC interaction, leading to the development of activated memory T cells locally and activation of the immune response, as well as the subsequent development of rheumatoid synovitis. After triggering inflammation of the synovium, memory T-cell activation leads to progression of inflammation in rheumatoid synovium, i.e., infiltration of inflammatory cells, increase of vascular vessels, formation of follicular germinal centers, and proliferation of synoviocytes (phase 2 in Fig. 6). Destructive inflammation then progresses to cartilage and bone injury by pannus (phase 3 in Fig. 6). As a result, progressive inflammation leads to synovial membrane invasion of bone, loss of cartilage and bone destruction in joints.

Molecular-targeted therapeutic strategies in RA

Although the specific antigens responsible for the pathogenesis of RA have not been identified, T-cell activation via interaction with APCs plays a pivotal role in disease development. In this regard, therapeutic strategies have targeted cellular pathways in RA. In addition to anti-cytokine reagents, impressive therapeutic effect has been recently reported following the blocking of CD28-mediated costimulation by the use of cytotoxic T-lymphocyte-associated antigen 4-IgG1 (CTLA4Ig).[84,85] Expressed on T cells within hours to days after activation,[86,87] CTLA4 is the high-avidity receptor for both CD80 and CD86, and inhibits T-cell proliferation and IL-2 production.[88,89] A fusion protein, CTLA4Ig binds both CD80 and CD86 on APCs, thereby preventing these molecules from engaging CD28 on T cells.[90] By blocking the engagement of CD28, CTLA4Ig prevents the delivery of the second costimulatory signal that is required for optimal activation of T cells. The successful usage of this agent therefore demonstrates that blocking costimulatory signal to inhibit T-cell activation is a novel and promising therapeutic concept for selected autoimmune diseases.[84,85] In this regard, since we showed that CD26–cavolin-1 interaction may play a pivotal role in rheumatoid synovitis, reagents capable of blocking CD26–cavolin-1 interaction in synovitis may be potentially useful in the treatment of patients with RA.







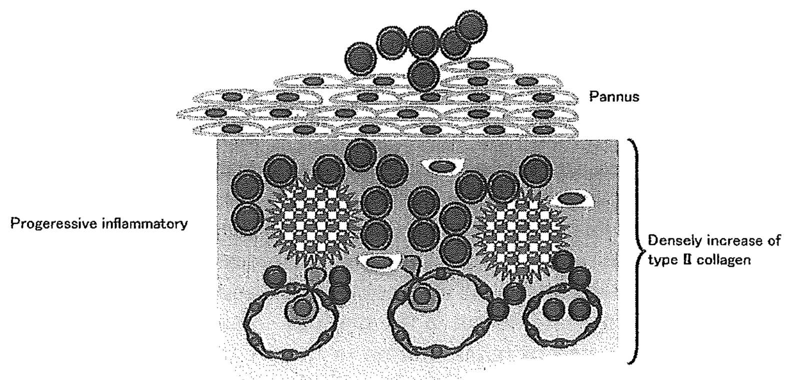


Fig. 6. Schematic diagram of inflammatory progress in rheumatoid synovitis. See text for details

Conclusions

Our results may thus provide a new approach to the treatment of autoimmune diseases or other immune-mediated disorders by directly intervening with the interaction between activated T-cell and APC. Targeting the binding of the pocket structure of CD26 and the scaffolding domain of caveolin-1 may lead to novel therapeutic approaches, including the utilization of antagonists that regulate antigen-specific immune response in immune-mediated disorders such as RA.

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HTLV-I Tax induces and associates with Crk-associated substrate lymphocyte type (Cas-L)

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Crk-associated substrate lymphocyte type (Cas-L) is a docking protein that is heavily tyrosine phosphorylated by the engagement of β1 integrins in T cells. In the present study, we attempted to evaluate the role of Cas-L in the pathophysiology of adult T-cell leukemia (ATL). Examination of peripheral blood mononuclear cells from ATL patients as well as ATL-derived T cell lines showed an elevation of Cas-L in these cells. We showed that tyrosine phosphorylation as well as expression of Cas-L was markedly elevated through the induction of human T-lymphotropic virus type I (HTLV-I) Tax in JPX-9 cells, with these cells showing marked motile behavior on the ligands for integrins. We next performed yeast two-hybrid screening of cDNA library from an HTLV-I-transformed T cell line, which resulted in the identification of Tax as a putative binding partner for Cas-L. Co-precipitation experiments revealed that the serine-rich region of Cas-L might serve as the binding site with the highest affinity for Tax. Co-localization study showed that Tax and Cas-L partly merged in the cytoplasm. Finally, we showed that exogenous Cas-L inhibited Tax-mediated transactivation of nuclear factor κB (NF-κB), while Tax-independent activation of NF-κB remained intact, hence indicating that Cas-L might specifically regulate Tax-NF-κB pathway.

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Introduction

It has been shown that β1 integrins exhibit a variety of biological functions such as cytokine production, proliferation, cell differentiation, cell survival, apoptosis, and cell migration as well as cell adhesion through specific interaction with their ligands (extracellular matrix and vascular cell adhesion molecule (VCAM)-1) (Hemler, 1990; Hynes, 1992; Juliano and Haskill, 1993; Ruoslahti and Reed, 1994). To understand the molecular mechanisms involved with these numerous biological effects, it is particularly important to analyse cell signaling through the β1 integrins. In this regard, we demonstrated that interaction of FN and very late antigen (VLA)-5 or the CS-1 domain of FN and VLA-4 could induce costimulatory signals to CD3/TCR pathway (Matsuyama *et al.*, 1989; Nojima *et al.*, 1990). Our subsequent study showed that PLC-γ, FAK (focal adhesion kinase), paxillin, fyn, lck, ERK1/2, and pp105 were tyrosine-phosphorylated upon engagement of β1 integrins in T cells (Nojima *et al.*, 1992; Sato *et al.*, 1995; Tachibana *et al.*, 1995). Isolation of cDNA encoding pp105 revealed that this protein belonged to Cas family, and we designated it Cas-L (Cas lymphocyte type) (Law *et al.*, 1996; Minegishi *et al.*, 1996).

Cas-L is a docking protein that is heavily tyrosine phosphorylated by FAK and Src family kinases upon engagement of β1 integrins as well as the TCR complex in T cells (Tachibana *et al.*, 1997; Ohashi *et al.*, 1998). Cas-L cDNA was cloned initially from adult T-cell leukemia (ATL)-derived T cell lines, and its expression was markedly elevated in those cells (Minegishi *et al.*, 1996). Recently, we have demonstrated that Cas-L was overexpressed and tyrosine phosphorylated *in vivo* in human T-lymphotropic virus type I (HTLV-I) Tax transgenic mice (Miyake-Nishijima *et al.*, 2003). These facts led us to evaluate the possible biological link among Cas-L, Tax, and HTLV-I-related disorders.

It is known that HTLV-I is the etiologic agent for ATL (Uchiyama *et al.*, 1977; Yodoi and Uchiyama, 1986; Yoshida, 1993). One of the characteristics of this malignancy is the infiltration of leukemic T cells into the skin and other organs. Although the precise mechanism of pathogenesis of ATL remains unclear, $p40^{tax}$ protein is thought to play a key role in HTLV-I-mediated

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transformation of T cells (Yoshida, 1993). Tax, encoded by the pX region of the HTLV-I genome, is a potent transactivator of various host genes as well as HTLV-I LTR-mediated transcription of the viral genome (Sodroski *et al.*, 1984). Tax activates transcription of numerous host cellular genes associated with lymphocyte proliferation, for examples, proto-oncogenes (Fujii *et al.*, 1991), cytokines and cytokine receptors including IL-2R (Inoue *et al.*, 1986; Maruyama *et al.*, 1987; Cross *et al.*, 1987), c-fos (Fujii *et al.*, 1988; Nagata *et al.*, 1989), TGF-β (Kim *et al.*, 1990), gp34/OX-40/CD134 (Miura *et al.*, 1991), IL-6 (Yamashita *et al.*, 1994), TNF-α (Albrecht *et al.*, 1992), IL-8 (Mori *et al.*, 1998), as well as the integrated provirus of HTLV-I (Sodroski *et al.*, 1984). These findings suggest its close association with T-cell immortalization and transformation. It has been shown that this transactivation of host genes is mediated through the interaction of $p40^{tax}$ with several cellular transcription factors, such as cyclic AMP-responsive element binding protein/activating transcriptional factor (CREB/ATF), NF-κB, and serum responsive factor (SRF) (Yoshimura *et al.*, 1990; Yoshida, 1993; Franchini, 1995; Harhaj and Sun, 1999).

In this paper, we demonstrate the possible involvement of Cas-L in the pathogenesis of ATL through Tax-mediated overexpression and hyper-phosphorylation of Cas-L, which resulted in markedly enhanced motility of lymphocytes. Furthermore, we showed that the interaction of Tax and Cas-L may result in the modulation of Tax-mediated transactivation of NF-κB.

Results

Predominant expression of Cas-L in HTLV-I-transformed T cell lines and ATL-derived T cell lines

We previously described and cloned pp105/Cas-L in ATL-derived T cell lines H9 (Nojima *et al.*, 1992; Minegishi *et al.*, 1996). In those cells, higher levels of Cas-L protein and mRNA were observed than unstimulated peripheral T cells. To investigate the distribution of Cas-L, a variety of HTLV-I-transformed T cell lines, ATL-derived T cell lines, and HTLV-I-unrelated T cell lines were examined for expression of Cas-L as well as FAK, Pyk2 (FAK-related tyrosine kinase), paxillin, and HTLV-I Tax. As shown in Figure 1, the expression of Cas-L was higher in the cases of HTLV-I-transformed T cell lines (MT-1, MT-2, and MT-4) and ATL-derived T cell lines (Hut-102, H9, and SLB-I) than HTLV-I-unrelated T cell lines (Jurkat and HPB-ALL). In contrast, expression of FAK, Pyk2, and paxillin, which are also involved in β1 integrin-mediated cell signaling, did not exhibit such distribution pattern among these T cell lines.

Elevation in protein level and tyrosine phosphorylation level of Cas-L and other related signal-transducing proteins downstream of β1 integrins in Tax-induced JPX-9 cells

Since our results showed that ATL-derived and HTLV-I transformed T cell lines expressed Cas-L at higher levels,

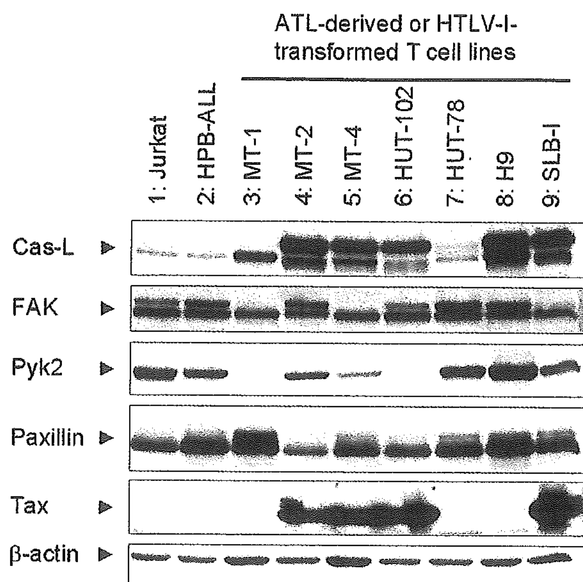

Figure 1 Expression of Cas-L in ATL-derived or HTLV-I-transformed T cell line and HTLV-I (−) T cell lines. Each indicated cell line was lysed in 1% NP-40 lysis buffer. Equal amounts of the whole-cell lysates (50 μg per lane) were separated on SDS–PAGE, and analysed by immunoblotting with anti-Cas mAb, anti-FAK mAb, anti-Pyk2 mAb, anti-paxillin mAb, anti-Tax mAb, and anti-β-actin mAb, respectively

we hypothesize that HTLV-I Tax protein might cause the preferential overproduction of Cas-L in HTLV-I-infected T cell lines. We examined the expression of Cas-L in JPX-9 cells and JPX-M cells, in which Tax protein is induced by a metallothionein promoter (Nagata *et al.*, 1989). JPX-M cells were used as negative controls, in which inactive mutant Tax protein was induced by the same stimuli. As shown in Figure 2a, Cas-L protein level was elevated in JPX-9 cells in a time-dependent manner after the addition of $CdCl_2$, whereas control JPX-M cells stimulated in the same condition did not show any alteration in Cas-L level.

We next compared the expression of Cas-L and other proteins involved in β1 integrin-mediated signaling by immunoprecipitation and immunoblotting. As shown in Figure 2b, the levels of FAK, paxillin, and Cas-L were also elevated following induction of Tax in JPX-9 cells. Surprisingly, levels of spontaneous tyrosine phosphorylation of Cas-L, FAK, Pyk2, and paxillin were also elevated by induction of Tax. In contrast, the levels of protein expression and tyrosine phosphorylation of these proteins were not altered in JPX-M cells.

Enhanced motility of Tax-induced JPX-9 cells

We previously reported that gene transfer of Cas-L provided Jurkat cells with motile behavior on FN-, CD3- plus FN-, and CS-1-coated surface, and that this enhancement of motility required the tyrosine phosphorylation of Cas-L (Ohashi *et al.*, 1999). We therefore evaluated the motility of JPX-9 cells and JPX-M cells on CS-1-coated Transwell™ insert. As shown in Figure 3a,

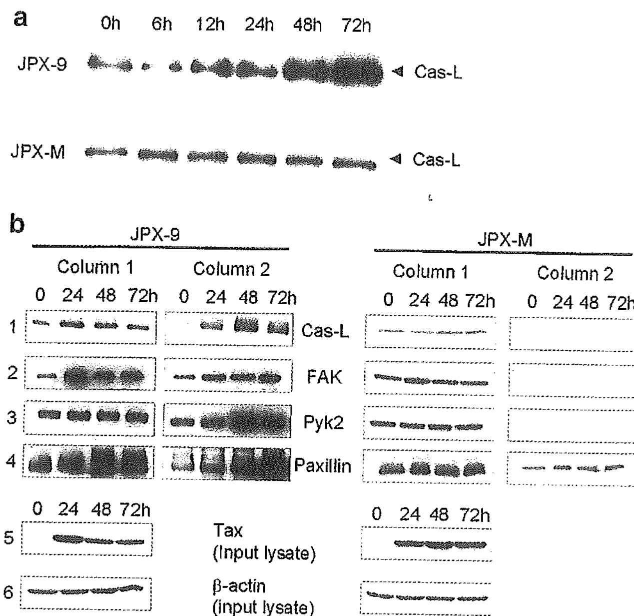

Figure 2 (**a**) Cas-L induction by $CdCl_2$ in JPX-9 cells. JPX-9 cells and JPX-M cells were cultured in the presence of $10\,\mu M$ $CdCl_2$ at 37°C for 6–72 h. Equal amounts of the whole-cell lysates ($50\,\mu g$ per lane) were separated on SDS–PAGE, and analysed by immunoblotting with anti-Cas mAb. (**b**) Protein amounts of Cas-L, FAK, Pyk2 and paxillin and their levels of tyrosine phosphorylation in Tax-induced JPX-9 cells and JPX-M cells. JPX-9 cells and JPX-M cells were cultured in the presence of $10\,\mu M$ $CdCl_2$ at 37°C for 24–72 h. In total, 1×10^7 cells were lysed in the 1% NP-40 lysis buffer and immunoprecipitated with anti-Cas-L Ab, anti-FAK mAb, anti-Pyk2 mAb, or anti-paxillin mAb, respectively. The precipitates were analysed by immunoblotting with anti-Cas mAb, anti-FAK mAb, anti-Pyk2 mAb, or anti-paxillin mAb, and reprobed with anti-phosphotyrosine mAb (4G10; pTyr)

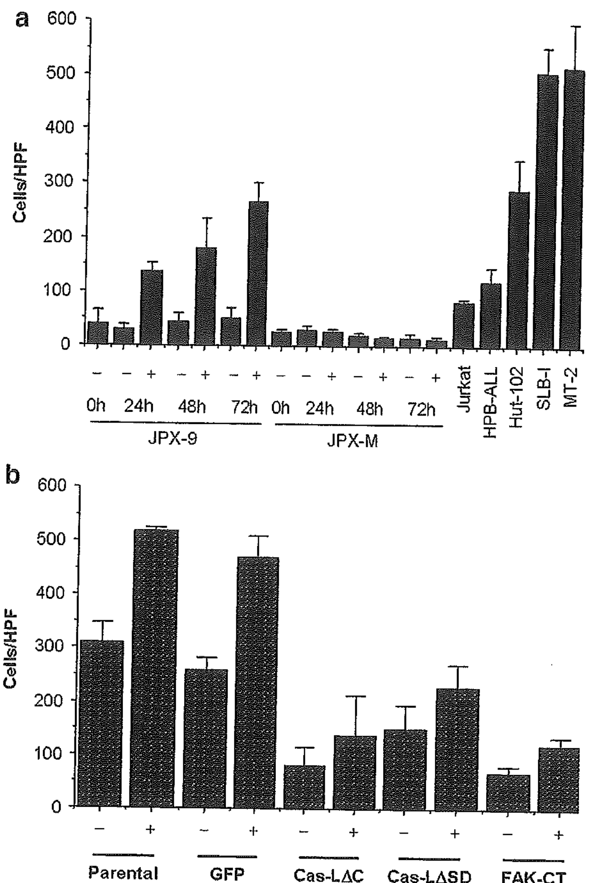

Figure 3 (**a**) Tax-induced cell motility on CS-1 in JPX-9 cells and JPX-M cells. JPX-9 cells and JPX-M cells were cultured with or without $10\,\mu M$ of $CdCl_2$ at 37°C for 24–72 h. Jurkat, HPB-ALL, Hut-102, SLB-I, and MT-2 cells were cultured in normal medium and harvested prior to the assay. The cells were incubated at 37°C for 4 h on the Transwell™ inserts coated with CS-1/GST fusion protein ($5\,\mu g$/ml). (**b**) Tax-induced cell motility on FN and CD3 mAb in JPX-9 cells infected with retrovirus coding for dominant-negative constructs of Cas-L or FAK. JPX-9 cells and those infected with each retrovirus (GFP: pMX-IRES-GFP, ΔC: pMX-Cas-LΔC-IRES-GFP, ΔSD: pMX-Cas-LΔSD-IRES-GFP, FAKCT: pMX-FAKCT-IRES-GFP) were cultured in the presence or absence of $CdCl_2$ for 48 h. Cells were incubated at 37°C for 4 h on the Transwell™ inserts coated with FN and CD3 mAb ($5\,\mu g$/ml each). Data are representative of two independent experiments (**a**, **b**)

JPX-9 cells stimulated with $CdCl_2$ displayed remarkable motility compared to unstimulated JPX-9 cells. In the control JPX-M cells, such promotion of motility by induction of Tax was not observed. As shown in the figure, the enhanced motility observed in Tax-induced JPX-9 cells was comparable to Hut-102, SLB-I, and MT-2, which showed higher motility than HTLV-I-unrelated T cell lines (Jurkat and HPB-ALL). To further characterize the cell motility observed in the Tax- and Cas-L induced JPX-9 cells, we transduced dominant-negative Cas-L (ΔSD and ΔC) or FAK-CT constructs by retroviral gene transfer. As shown in Figure 3b, gene transfer of these constructs resulted in the reduced induction of motility on CD3 plus FN-coated surface, indicating that Tax-mediated enhancement of T cell motility was at least partly mediated through $\beta 1$ integrins–Cas-L pathway.

Enhanced expression and tyrosine phosphorylation of Cas-L in PBMCs from ATL patients

To further explore the clinical relevance of Cas-L overproduction in HTLV-I-related diseases, we obtained samples from ATL patients as well as asymptomatic HTLV-I carriers. As shown in Figure 4, mononuclear cells from the peripheral blood of ATL patients had elevated expression of Cas-L in four of five cases examined. Interestingly, the levels of tyrosine phosphorylation of Cas-L were clearly elevated in two of those cases (#4 and #8). In contrast, neither overproduction of Cas-L nor enhanced phosphorylation of Cas-L was observed in the cases with asymptomatic HTLV-I carriers. Thus, overproduction of Cas-L and, in some cases, elevation of its tyrosine phosphorylation was also observed in ATL patients.

Identification of Tax as a putative Cas-L binding protein

To further understand the biological significance of Cas-L in ATL and HTLV-I related disorders, we next performed two-hybrid cDNA cloning in search for Cas-L binding proteins. We screened over 1×10^6 indepen-

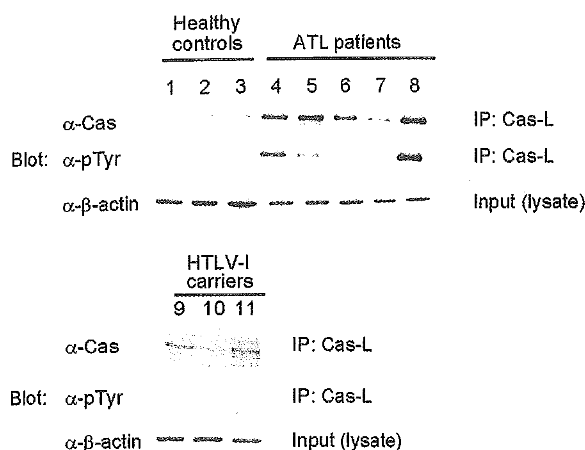

Figure 4 Expression and tyrosine phosphorylation of Cas-L in PBMCs from ATL patients and HTLV-I carriers. Leukemic cells from five patients diagnosed with acute-type ATL (lanes 4–8), and PBMCs from HTLV-I infected individuals (lanes 9–11) and healthy controls (lanes 1–3) were analysed. The diagnosis of ATL and HTLV-I carrier was based on clinical features, hematologic findings, and the presence of anti-HTLV-I antibodies in patient sera. Equal amounts of the whole-cell lysates (25 μg per lane) were separated on SDS–PAGE, and analysed by immunoblotting with anti-Cas mAb (α-Cas; the upper panel), then reprobed with anti-phosphotyrosine mAb (α-pTyr; the middle panel). As controls, immunoblotting with anti-β-actin mAb was carried out (α-β-actin; the bottom panel)

dent clones from the cDNA library of an HTLV-I infected T cell line, SLB-I. Among 180 clones selected by histidine prototrophy and β-Gal expression, we were especially interested in two independent Tax clones, one (clone #80) of which contained almost full-length (aa 2–353) Tax and the other (clone #45) of which contained the c-terminal half (aa 192–353) of the Tax cDNA in frame.

Co-precipitation of Cas-L and p130Cas with Tax

To evaluate protein–protein interaction of Cas-L and Tax, we performed co-precipitation experiments using 293T cells. Initially, we employed full-length constructs for Cas-L and p130Cas, another Cas family member. As shown in Figure 5a, transfected Cas-L was co-precipitated with Tax in those cells. Since p130Cas was the first reported Cas family protein with well-conserved domain structures, we performed the same studies as those done with Cas-L, and found that p130Cas was similarly co-precipitated with Tax.

We also performed the co-precipitation experiments using MT-2 and Hut-102 cells that co-express Cas-L and Tax. As shown in Figure 5b, Cas-L protein was co-precipitated with Tax in those cells, suggesting that endogenous Cas-L and Tax associated with each other in HTLV-I-infected T cell lines.

Determination of Tax-binding domains of Cas-L

To identify the Tax-binding domain of Cas-L, we first employed a series of deletion mutants: Cas-LΔSH3: lacking SH3 domain (aa 1–60); ΔSD: lacking substrate domain (aa 63–401); and ΔC: lacking c-terminal half (aa 406–834). We also prepared a point mutant Cas-LF, in which the Src-SH2-binding motif YDYVHL was mutated to FDFVHL (Tachibana *et al.*, 1997). The structures of these mutants were schematically summarized in Figure 5c. Unexpectedly, none of these mutations or deletions could abolish the binding capability of Cas-L with Tax in 293T cells (Figure 5d), implying multiple binding sites were present in Cas-L, as was suggested in the case involving the association of Cas-L/HEF1 and Smad3 (Liu *et al.*, 2000). We then produced GST-fusion protein of each domain. As shown in Figure 5e, SR or SRF had equivalent and the strongest binding capability. In addition, SD and HLH showed significant binding with Tax, whereas SH3 showed no binding capability.

Co-localization of Cas-L with Tax in the cytosol of transfected 293T, MT-2, and Hut-102 cells

We next determined the co-localization of Cas-L with Tax by confocal microscopy. As shown in Figure 6A, transfected c-myc-tagged Cas-L alone mainly localized in the cytosol (column a, row 2), which was not altered by the co-transfection of Tax (column a, row 1). In contrast, transfected Tax localized both in the nucleus and cytosol (nucleus dominant; column b, row 3), which was changed to a cytosol dominant pattern (column b, row 2). This alteration of subcellular localization was observed especially in cells co-expressing Cas-L and Tax (column c, row 1). Furthermore, Cas-L and Tax were mainly co-localized in the cytosol (column c, row 1). Similar results were also observed in the cases with Hut-102 and MT-2 cells, in which Cas-L and Tax proteins were endogenously co-expressed (Figure 6B). These results suggest that Cas-L and Tax possibly cooperate by protein–protein interaction mainly in the cytosol.

Exogenous Cas-L interferes with Tax-mediated transactivation of NF-κB

Tax is known as a potent transcriptional activator of host genes flanked by κB sequences as well as HTLV-I LTR. We examined the effect of Cas-L on Tax-associated κB- or TRE-dependent transactivation. As shown in Figure 7a, co-transfected Cas-L partially, but potently, inhibited Tax-mediated activation of an NF-κB-dependent transcription. This inhibitory activity was observed in all deletion mutants examined (Cas-LF, ΔSH3, ΔC, ΔSD), although the activity was weakest in Cas-LΔC, which is consistent with the co-immunoprecipitation assay (Figure 5d and e). On the other hand, Tax-mediated transcriptional enhancement of TRE-luciferase was not altered (Figure 7b). To assess the specificity of inhibition of κB-driven transcriptional activation, we next examined the effect of Cas-L on TNF-α (on 293T cells) or PMA (on Jurkat T cells)-stimulated activation of NF-κB. As shown in Figure 7c and d, expression of Cas-L or p130Cas did not affect NF-κB activation by these stimuli. These results strongly suggest that exogenous Cas-L specifically interferes with Tax-mediated activation of NF-κB.

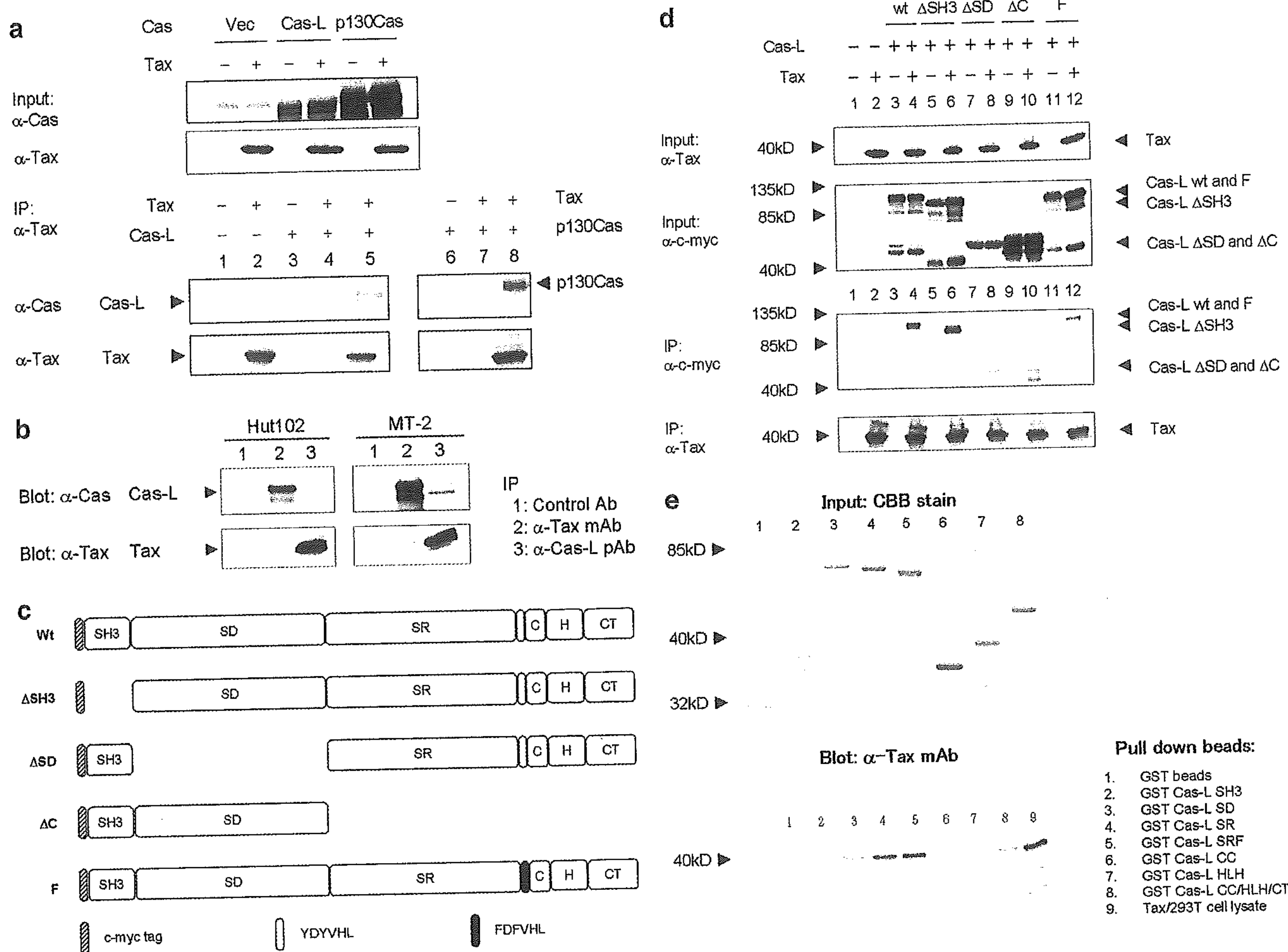

Figure 5 (a) Co-precipitation of Cas-L and p130Cas with Tax in 293T cells. 293T cells were transfected with pH2Rneo (−) or pH2Rneo-Tax (+), in the presence or absence of pSRalpha (Vec), pSRalpha Cas-L (Cas-L), or pSRalpha p130Cas (p130Cas). At 2 days after transfection, the cells were lysed with 1% Triton X-100 lysis buffer. *Input*: The lysates of 293T cells transfected with the indicated combination of plasmids were separated on SDS–PAGE, and subjected to immunoblotting with α-Cas mAb and α-Tax mAb. *IP*: Equal amounts of cellular lysates of 293T cells transfected with the indicated combination of plasmids were immunoprecipitated with isotype-matched control Ig (lanes 4, 7), anti-Tax mAb (lanes 1–3, 5, 6, 8). The precipitates were analysed by immunoblotting with anti-Cas mAb or anti-Tax mAb, respectively. (b) Co-precipitation of endogenous Cas-L and Tax in HTLV-I positive T cell lines. In toal, 1×10^7 of MT-2 cells and Hut102 cells were lysed with 1% Triton X-100 lysis buffer, then immunoprecipitated with the indicated antibodies. The immunoprecipitates were analysed by immunoblotting for anti-Cas mAb (the upper panel), and anti-Tax mAb (the lower panel). (c) Structure of Cas-L and its mutants. The secondary structure of Cas-L was graphically shown. The c-myc tag (MEQKLISEEDL) inserted 5′ prime to the second amino acid of Cas-L was visualized as dashed box. From 5′ prime, SH3: Src homology 3 domain, SD: substrate domain, SR: serine-rich region, C: Coiled-coil domain, H: helix–loop–helix domain, CT: c-terminal region. (d) Co-precipitation of Cas-L mutants with Tax in 293T cells. 293T cells were transfected with the indicated c-myc tagged Cas-L constructs (wt: pSRalpha c-myc Cas-L; ΔSH3: pSRalpha c-myc Cas-LΔSH3; ΔSD: pSRalpha c-myc Cas-LΔSD; ΔC: pSRalpha c-myc Cas-LΔC; F: pSRalpha c-myc Cas-LF; Vec: pSRalpha) either with pH2Rneo (−) or pH2RneoTAX (+). The top panel (*Input*): Equal amounts of cellular lysate from each transfected 293T cells were separated on SDS–PAGE and immunoblotted with anti-c-myc mAb. The middle panel (*α-c-myc*): Equal amounts of cellular lysate were immunoprecipitated with anti-Tax mAb, then analysed by immunoblotting with anti-c-myc mAb. The lower panel (*α-Tax*): The same membrane as the middle panel was reblotted with α-Tax mAb. (e) GST-pulldown assay with Cas-L mutants and Tax. The cellular lysates containing Tax were prepared by transfecting pH2Rneo-TAX by lipofectamine reagent. The lysates were incubated with recombinant GST-Cas-L domains immobilized on GSH-sepharose beads. The upper panel: Input of GST-fusion proteins were separated on SDS–PAGE, and visualized by CBB stain. Co-precipitated Tax proteins were separated on SDS–PAGE and subjected to immunoblotting with anti-Tax mAb (the lower panel). SRF: serine-rich region with FDFVHL, CC: coiled-coil domain, HLH: helix–loop–helix domain

Discussion

In the present study, we have shown that HTLV-I Tax may induce the expression and tyrosine phosphorylation of Cas-L. Using Jurkat T cells, we previously showed that gene transfer of Cas-L promoted cell motility on the ligands for $\beta 1$ integrins (i.e. FN and CS-1) in the presence or absence of anti-CD3 mAb stimulation (Ohashi *et al.*, 1999). This enhancement was dependent on tyrosine phosphorylation of Cas-L, since the phosphorylation-deficient mutant of Cas-L (ΔSH3 and ΔSD) failed to enhance cell migration. Tax-induced JPX-9 cells clearly exhibited elevated level of tyrosine phosphorylation as well as elevated expression level of

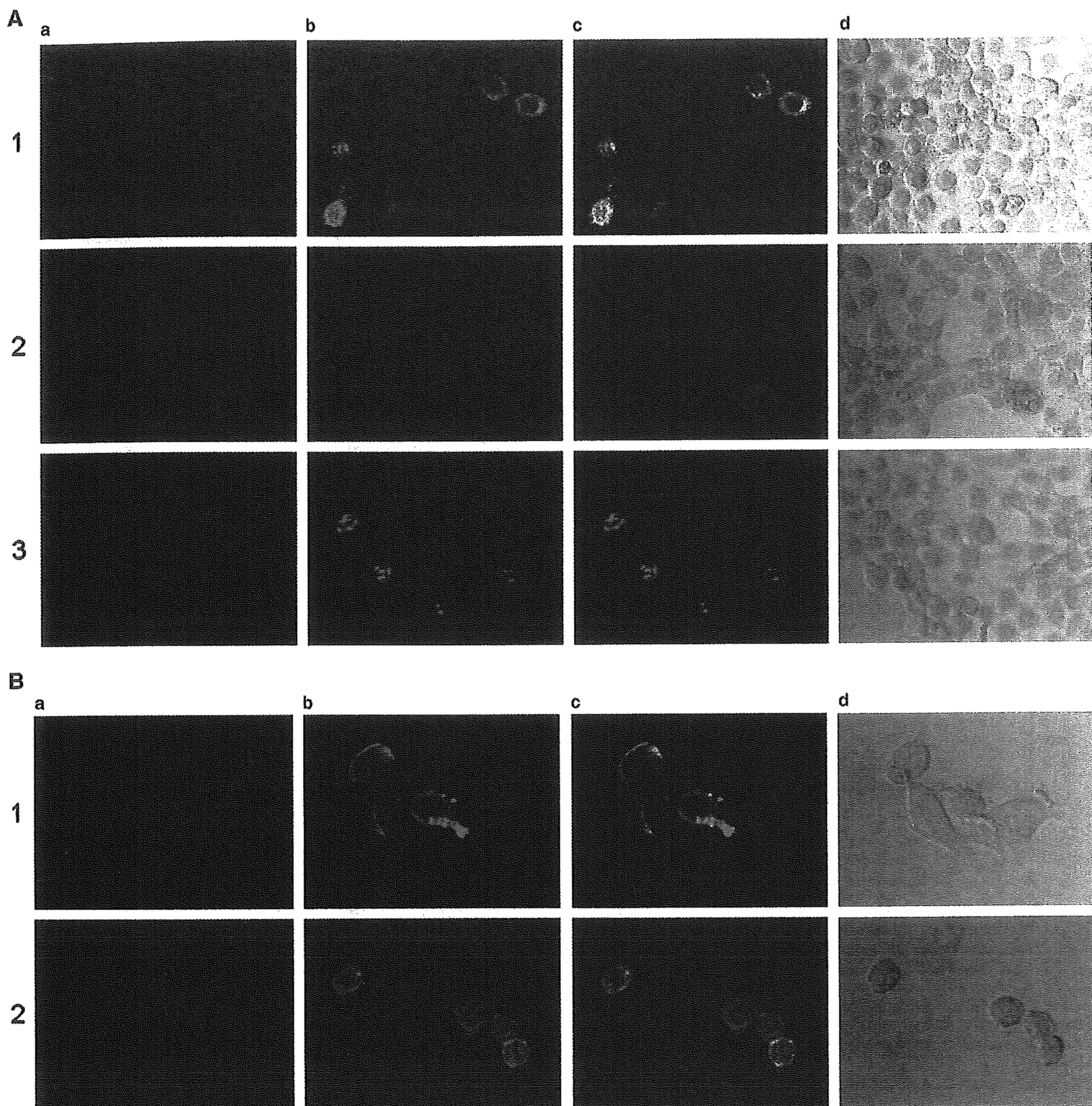

Figure 6 Co-localization of Cas-L with Tax in 293T cells. (**A**) 293T cells were transfected with pSRalpha c-myc Cas-L and pH2Rneo-Tax (row 1), pSRalpha c-myc Cas-L and pH2Rneo (row 2), pSRalpha and pH2Rneo-Tax (row 3) by lipofectamine on the glass coverslips. At 48 h after transfection, the cells were fixed and subjected to immunostaining. Subcellular localization of transfected proteins was analysed with confocal microscopy. Column a: Cy3-conjugated anti-c-myc mAbs; column b: anti-Tax mAb plus FITC-conjugated anti-mouse Ab; column c: merged results of the red (a) and green fluorescence (b) in the sequential mode; column d: phase contrast. (**B**) Hut-102 cells (row 1) and MT-2 cells (row 2) were cultured on the glass coverslips coated with FN at 5 μg/ml for overnight. The cells were fixed and subjected to immunostaining and confocal microscopic analysis. Column a: rabbit anti-CasL Ab plus FITC-conjugated anti-rabbit Ab; column b: anti-Tax mAb plus Texas Red-conjugated anti-mouse Ab; column c: merged results of the red (a) and green fluorescence (b) in the sequential mode; column d; phase contrast

Cas-L. These findings are of special interest since leukemic cells in ATL are notorious for extensive infiltrative activity toward organs and tissues, contributing to the unique clinical features of ATL (Uchiyama, 1996). A variety of molecules involved in cellular adhesion and migration are thought to play important roles in ATL pathophysiology, including MMP-9 (Mori *et al.*, 2002), OX-40 (Imura *et al.*, 1996), β1 integrins (especially VLA-4) (Ishikawa *et al.*, 1993), and L-selectins (Ishikawa *et al.*, 1993). Our current results

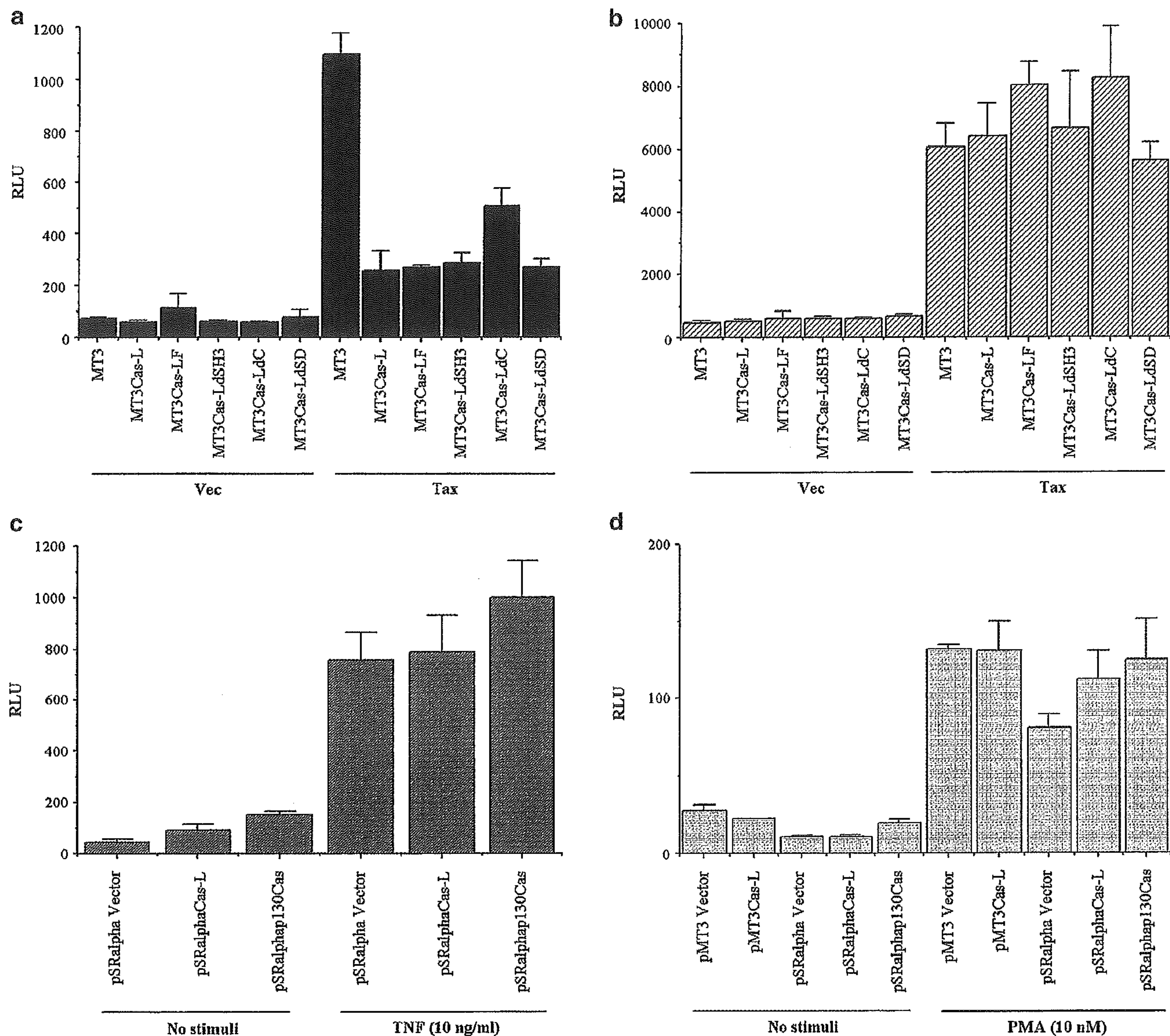

Figure 7 Effect of Cas-L expression on transactivation by Tax. (**a**, **b**) Jurkat-LT cells were transfected with NF-κB-Luc (**a**) or TRE-Luc (**b**), the indicated expression plasmids for wt (wild type) Cas-L, various Cas-L mutants, or pMT3 either with pH2Rneo (Vec) or pH2Rneo TAX by DMRIE-C. (**c**) 293T cells were transfected with NF-κB-Luc, the indicated plasmids for Cas-L, p130Cas, or pSRalpha by Lipofectamine. The cells were simultaneously stimulated with the indicated concentration of TNF-α or PMA. (**d**) Jurkat-LT cells were transfected with NF-κB-Luc, the indicated plasmids for Cas-L, p130Cas, or vector control (pSRalpha or pMT3) by DMRIE-C. The cells were simultaneously stimulated in the presence or absence of 10 ng/ml PMA. After 24 h, the lysates were subjected to luciferase assay (**a–d**)

imply that Cas-L may be one of targets for therapeutic intervention through modulation of its overexpression or tyrosine phosphorylation. Our results that the dominant-negative Cas-L mutants and FAK-CT inhibited Tax-mediated increase in cell motility indicating the involvement of Cas-L. However, it is still possible that some other Tax-inducible genes such as those described above might be involved in this phenomenon. The precise mechanism by which Cas-L was tyrosine-phosphorylated through the expression of Tax is an important question that remains to be elucidated.

It should be noted that the expression levels of Tax and Cas-L did not perfectly correlate in HTLV-I-transformed T cell lines and ATL-derived (MT-1 and H9) and patients samples from ATL. Indeed, in those clinical samples, the expression of Tax was hardly detected by immunoblotting (data not shown). This was consistent with the report that expression of Tax and viral mRNAs is tightly regulated *in vivo* by HTLV-I itself to escape from host defense mechanisms involving cytotoxic T lymphocytes reactive for Tax and env protein of HTLV-I (Yoshida, 1993). Therefore, the possibility exists that other undefined factors may contribute to the elevated expression of Cas-L *in vivo*.

In this study, we identified Tax as one of the Cas-L binding molecules. Others and we have shown that Cas