Human T Lymphotropic Virus Type 1 Increases T Lymphocyte Migration by Recruiting the Cytoskeleton Organizer CRMP2

Michel Varrin-Doyer, Adeline Nicolle, Romain Marignier, Sylvie Cavagna, Claire Benetollo, Eric Wattel and Pascale Giraudon

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Lymphocyte infiltration in the CNS is at the basis of neuroinflammatory diseases, including those associated with virus infection. Besides CNS infection, infected immune cells can affect neurons and glial cells through the bystander effect of secreted inflammatory molecules, leading progressively to demyelination and axonal loss (1). Dissection of the complex mechanisms that control infiltration and migration of infected T lymphocytes into the CNS is of importance in understanding the pathophysiology of virus-induced neuroinflammatory diseases. One prototype of such a disease is human T cell lymphotropic virus type-1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP). This human retrovirus mainly targets CD4 and CD8 T lymphocytes and is the etiological agent of both adult T cell leukemia (ATL) (2) and HAM/TSP (3). The predominant viral reservoir in the peripheral blood is CD4+CD25+ T cells (4). How HTLV-1 causes neurologic disorders is not completely understood, but several works have focused on the crucial role of infected T cells in pathogenesis. It is thought that, in HAM/TSP patients, HTLV-1–infected lymphocytes migrate from the periphery into the CNS, because lymphocytes of the cerebrospinal fluid and PBMC shared the same HTLV-1 integration site in cellular DNA (5). Detection of HTLV-1 genome and the viral protein Tax in inflammatory T lymphocytes infiltrated in the patients’ CNS has identified T lymphocytes as the main virus reservoir in the brain and the main effectors of pathological changes (6–10). In fact, Tax has the ability to promote or alter the expression and activity of several cellular factors involved in T cell behavior (11–13), as such, may contribute to pathogenic mechanism. In this context, we have previously shown that HTLV-1–infected T cells disturb the metabolism of astrocytes through the activity of TNF-α (14). Secreted by infected T lymphocytes, TNF-α reduced the glutamate transporter EAAT2/GLT1 at astrocyte membrane and, consequently, increased the extracellular level of glutamate, an excitotoxic amino acid deleterious for neurons and oligodendrocytes at a high level. Other virus proteins are also involved in pathogenesis, notably the p8 protein, that promote T cell contact and enhance virus transmission (15). Given the crucial role of infected T lymphocytes in pathogenesis, facilitation of their recruitment into the CNS through changes induced by HTLV-1 in the cell machinery of locomotion could be an important phase of the disease.

**Human T Lymphotropic Virus Type 1 Increases T Lymphocyte Migration by Recruiting the Cytoskeleton Organizer CRMP2**

Michel Varrin-Doyer,*† Adeline Nicolle,*† Romain Marignier,*† Sylvie Cavagna,*† Claire Benetollo,*† Eric Wattel,‡ and Pascale Giraudon*†

Recruitment of virus-infected T lymphocytes into the CNS is an essential step in the development of virus-associated neuroinflammatory diseases, notably myelopathy induced by retrovirus human T leukemia virus-1 (HTLV-1). We have recently shown the key role of collapsin response mediator protein 2 (CRMP2), a phosphoprotein involved in cytoskeleton rearrangement, in the control of human lymphocyte migration and in brain targeting in animal models of virus-induced neuroinflammation.

Using lymphocytes cloned from infected patients and chronically infected T cells, we found that HTLV-1 affects CRMP2 activity, resulting in an increased migratory potential. Elevated CRMP2 expression accompanies a higher phosphorylation level of CRMP2 and its more pronounced adhesion to tubulin and actin. CRMP2 forms, a full length and a shorter, cleaved one, are also affected. Tax transfection and extinction strategies show the involvement of this viral protein in enhanced full-length and active CRMP2, resulting in prominent migratory rate. A role for other viral proteins in CRMP2 phosphorylation is suspected. Full-length CRMP2 confers a migratory advantage possibly by preempting the negative effect of short CRMP2 we observe on T lymphocyte migration. In addition, HTLV-1–induced migration seems, in part, supported by the ability of infected cell to increase the proteosomal degradation of short CRMP2. Finally, gene expression in CD69+ cells selected from patients suggests that HTLV-1 has the capacity to influence the CRMP2/PI3K/Akt axis thus to positively control cytoskeleton organization and lymphocyte migration. Our data provide an additional clue to understanding the infiltration of HTLV-1–infected lymphocytes into various tissues and suggest that the regulation of CRMP2 activity by virus infection is a novel aspect of neuroinflammation. The Journal of Immunology, 2012, 188: 1222–1233.
During neuroinflammation, leukocytes emigrate from the bloodstream to the CNS across the blood–brain barriers through either endothelial cells of inflamed meningeal veins or epithelial cells of the choroids plexuses (16, 17). The continuous T lymphocyte extravasation, migration into, and crawling within neural tissue are orchestrated by the interplay of multiple adhesion receptors whose expression and avidity are modulated by cytokines and chemokines (18–20). Activation of G protein–coupled receptor by chemokines induces a motile phenotype characterized by the formation of lamellipodia at the front of migration and uropod at the rear of T lymphocyte (21, 22). In parallel, a drastic reconfiguration of the microtubule and intermediate filament cytoskeletons allows lymphocyte locomotion. However, the mechanism associated with lymphocyte motility per se is not totally defined. To extend our knowledge in this field, we recently focused our work on the phosphoprotein collapsin response mediator protein 2 (CRMP2), first described to modulate the microtubule reorganization during neural growth cone advance under semaphorin signal (23). Our experiments have highlighted the importance of CRMP2 in immune cells, notably in migratory activity, in both physiological and neuroinflammatory situations. We showed that CRMP2 was expressed in virtually all PBMC and had a crucial role in T lymphocyte polarization and migration (24). Investigation of the molecular mechanisms underlying CRMP2 activity in T lymphocyte indicated that CRMP2 transduced CXCL12/SDF1 chemokine signal. Differential phosphorylation by glycogen synthase kinase-3β and Yes was supposed to modulate the contribution of CRMP2 to cytoskeletal reorganization during chemokine-induced T cell migration (25). In addition, we showed that in mouse models of virus-induced neuroinflammation, the elevated CRMP2 expression in blood lymphocytes correlated with CNS infiltration and clinical signs, suggesting the potential use of CRMP2 as a peripheral indicator of neuroinflammation (26). Extending our analysis to human, we detected high CRMP2 expression in T lymphocytes of HTLV-1–infected patients suffering from neurologic disease compared with asymptomatic virus carriers (24). We now examined whether and how HTLV-1 modifies T cell motility through alteration of CRMP2 activity. The present data demonstrate that the virus enhanced T lymphocyte migration by sustaining the presence of active CRMP2 form in infected cells, in part through Tax activity. More generally, we point to the mechanisms exploited by a virus to intensify, in infected patients, the migratory potential of T lymphocyte.

Materials and Methods

Cells

Study was performed on CD4+ T cell clones generated by plating PBMC from two HTLV-1–infected patients (0.1 cell/well, 1 × 10^5, MT2, and MT4. T cell lines were cultured without IL-2. Cell selection of HD and HAM/TSP patients using MACS beads (Miltenyi Biotec). A total of 10 × 10^6 PBMCs were collected and centrifuged only four times before use. PBMCs were cultured in RPMI-1640 medium containing 10% fetal bovine serum and stained with Abs. Staining and scanning were performed in PBS-DakoCytomation. Cloned T cells were incubated with 5% filtered human serum and stained with specific Ab and then revealed by the ECL technology (Köln, Germany), according to the manufacturer’s instructions. T cells were transferred with unamplified RNA, or for quantitative PCR (qPCR) analysis on unamplified RNA, or for transmigration assays. either for RNA amplification and microarray analysis, for quantitative PCR (qPCR) analysis on unamplified RNA, or for transmigration assays.

Antibodies

CRMP2-A/B, generally termed CRMP2, was detected using purified polyclonal rabbit Abs directed against peptide 4 (ppe4) and C-ter epitopes, as previously described (29) and monoclonal C4G Ab from IBL-Japan. C4G and Tax Abs recognized only the full-length CRMP2. CRMP2-Δpep4 Ab recognized both the full-length (62 kDa) and cleaved (58 kDa) CRMP2. Polyclonal anti-CRMP2-A Ab was described previously (29). Ab specific of phosphorylated CRMP2 forms CRMP2-pSer522 and CRMP2-pThr509/514 was from Kinaseas (Dundee, U.K.); anti-CRMP2-pγT479 was produced as described previously (25). Anti-GAPDH Ab was from Chemicon. Ab to vimentin was from Calbiochem (IF01), to tubulin from Sigma-Aldrich (T6606), and to actin was from Santa Cruz Biotechnology (SC8432). Ab to Tax was from the National Institutes of Health (158A51-42). Small interfering RNA (siRNA) anti-CRMP2 and control were used as described previously (24).

Flow cytometry

Cells were washed in PBS and incubated (15 min at room temperature) with PE-labeled anti-CD69 (BD Pharmingen) or anti-CD4 (BD Biosciences) PhC and anti-CD45RA 5-labeled Ab (Biolegend). Cells were then washed in PBS containing 2% FCS. Isotype-matched controls were used. Data were acquired on the phosphoprotein collapsin response mediator protein 2 (CRMP2), first described to modulate the microtubule reorganization during neural growth cone advance under semaphorin signal (23). Our experiments have highlighted the importance of CRMP2 in immune cells, notably in migratory activity, in both physiological and neuroinflammatory situations. We showed that CRMP2 was expressed in virtually all PBMC and had a crucial role in T lymphocyte polarization and migration (24). Investigation of the molecular mechanisms underlying CRMP2 activity in T lymphocyte indicated that CRMP2 transduced CXCL12/SDF1 chemokine signal. Differential phosphorylation by glycogen synthase kinase-3β and Yes was supposed to modulate the contribution of CRMP2 to cytoskeletal reorganization during chemokine-induced T cell migration (25). In addition, we showed that in mouse models of virus-induced neuroinflammation, the elevated CRMP2 expression in blood lymphocytes correlated with CNS infiltration and clinical signs, suggesting the potential use of CRMP2 as a peripheral indicator of neuroinflammation (26). Extending our analysis to human, we detected high CRMP2 expression in T lymphocytes of HTLV-1–infected patients suffering from neurologic disease compared with asymptomatic virus carriers (24). We now examined whether and how HTLV-1 modifies T cell motility through alteration of CRMP2 activity. The present data demonstrate that the virus enhanced T lymphocyte migration by sustaining the presence of active CRMP2 form in infected cells, in part through Tax activity. More generally, we point to the mechanisms exploited by a virus to intensify, in infected patients, the migratory potential of T lymphocyte.
10^6 cells/well) were added in the upper chamber and incubated at 37°C for 1 h and 30 min. Chemokines CXCL12 (20 ng/ml), CCL5 (100 ng/ml), and CCL2 and CXCL10 (20 ng/ml) were added in the lower compartment. The migratory T cells in the lower chambers were counted under microscopy (at least 10 fields). Data are expressed as the mean number of migratory T cells.

Real-time PCR

RNA isolation was performed using the RNeasy PLUS (Qiagen), and residual genomic DNA was removed using Dnase I (DNase-free, Ambion), and reverse transcription was performed [500 ng total treated RNA and 100 ng oligo (dT)12–18 primers (33)]. cDNA was amplified by real-time PCR (LightCycler; Roche) using FastStart DNA Master SYBR Green I (Roche). PCR reactions were performed using Taq, CRMP2, and β-actin primers as follows: 15 min at 95°C (20 s, 95°C; 20 s, 60°C; and 10 s, 72°C), 48 cycles. Tax primers (J02029) were 5′-ATCCGGTACGAGCTCTCAAA-3′ and 5′-AACACGTGAGCTGGTATCC-3′. Human β-actin primers (NM_001101) were 5′-AAGAAGAGTCGACGAGTGAC-3′ and 5′-GGGCATGCTTGGAGATCTCAA-3′. Specificity of amplification was achieved by a final melting step giving a single melting peak at 83.4, 77.2, and 62°C for Tax, β-actin, and CRMP2 PCR product, respectively. The level of Tax and CRMP2 mRNA was expressed as relative units normalized to β-actin.

Gene expression profile

A transcriptomic approach was performed by the genomic platform facility ProfileXpert (Lyon, France; http://www.profilexpert.fr) on CD69+ selected cells using oligonucleotide microarrays (CodeLink Uniset human 20k bioarrays; General Electric Health Care) and Assistant Pathway software, as reported previously (34). Of the 19,881 known genes and EST surveyed on each array, 5,534 were expressed on the two chips for each human group (two HD; Etablissement Francais du Sang; two HAM/TSP patients; A. Gessain, Pasteur Institute, Paris, France). These latter represented the genes commonly expressed in CD69+ selected cells, whatever the clinical status. Only those coding for cluster of differentiation (CD), cytokine, chemokine, and their receptors were shown (Supplemental Table I). In contrast, some genes were exclusively expressed or displayed augmented expression in infected patients (Table I). Validation of data was performed as follows. Data from microarray analysis were validated on the same RNA preparation using quantification by real-time qPCR of 11 genes selected for their specific behavior (present, absent, or upregulated, in each group). In each case, results corroborated the microarray data. The specific expression of genes in CD69+ selected cells was confirmed using qPCR analysis of eight present genes and performed on mRNA isolated from CD69+ selected cells of new human group (two HAM/TSP and two HD). Again, results corroborated observations in the microarray approach. Microarray data are in the public database Gene Expression Omnibus (Series record GSE33859; http://www.ncbi.nlm.nih.gov/geo/).

Statistical analysis

Statistical significance in comparing two means was tested with the unpaired Student t test, and p < 0.05 was considered significant (in figures *p < 0.001 and **p < 0.01).

Results

High migratory rate of blood T lymphocytes isolated from HTLV-1–infected patients correlates with virus infection, activation status, and elevated CRMP2 expression

We previously reported that CRMP2 expression was elevated in total PBMC of HTLV-1–infected patients, notably in CD4+CD69+, CD45RO+, HLA-DR+, and VLA4+ lymphocytes (24). To investigate the role of a virus in CRMP2 modulation and its consequences on T cell migration, we now studied T lymphocytes isolated from infected patients and clonally established T lymphocytes in culture (termed T cell clones). Among the 18 T cell clones studied, 12 harbored the virus and expressed mRNA coding for the viral protein Tax. First, we analyzed by flow cytometry the frequency of CRMP2+ cell in T cell clones. It must be noted that CRMP2 is generally expressed in almost all T lymphocytes analyzed ex vivo, but the frequency of CRMP2+ cells is downregulated in vitro following culture. Virus-infected T cell clones displayed 75 ± 12% CRMP2+ cells compared with 34 ± 13% in noninfected clones (p < 0.001) (Fig. 1A), indicating that the CRMP2 expression level correlated with the infection status of T cell clones. Because HTLV-1 is known to chronically activate infected T lymphocytes, CRMP2 was analyzed in cells expressing CD69, a marker of T cell activation. Infected T cell clones displayed an elevated frequency of CD69+ cells (72 ± 4 versus 22 ± 3% in uninfected clones). Colabeling indicated that almost all activated T cells expressed CRMP2 (91 ± 6%) (Fig. 1B). Thus, CRMP2 expression level correlated with the activation status of T cell clones. This corroborated our previous analysis on PBMC of HTLV-1–infected patients, linking the high CD69+ cells frequency with elevated CRMP2 expression (24). To go deeper into the cell locomotion machinery of activated T lymphocytes during HTLV-1 infection, we analyzed the association between CRMP2 expression (cytometry), migratory rate (Transwell system), and gene expression profile (transcriptome) in activated cells selected from HAM/TSP patients and HD (selection on CD69 Ag). CRMP2 was higher in selected cells of patients (mean fluorescence intensity = 72.2 ± 2 versus 33.3 ± 10 in HD) and migratory rate specifically elevated (22 ± 2 versus 14 ± 2 migrating cells per hour for HD; p < 0.02). Gene expression analysis (Supplemental Table I) revealed the main presence of T lymphocytes in selected cells (CD2, CD38γc, CD7, and CD8αβ mRNA) and their activation status (CD69, CD96, and CD97). Dendritic cell markers were also detected (CD1c,d, CD58, CD86, and CD207). These two cell types are susceptible to HTLV-1 infection and have been detected in HAM/TSP patients (Ref. 35 and reviewed in Ref. 36). Several genes were associated with immune cell homing (CXCL2, CXCL8, CCL4L, CCL5, CCL22, CXCL10, CXCL16, CCR6, and CCR7) and transendothelial migration (CXC4, CD18, CD44, CD49a/VLAA4, CD58, and CD164). HAM/TSP patients exhibited a distinctive profile, with 22 genes uniquely expressed and 10 others with a ≥3-fold increased expression (Table I). Elevated expression of CD69 and HLAG confirmed that lymphocytes were highly activated in HAM/TSP. In addition, several induced genes were involved in molecular pathways acting downstream chemokine receptors, notably G protein and PI3K signaling (ARHGAP8, GNA13, GRASP, GRK5, GPR68, PIK3C2A, PI3KRI, RGS1, and RGS16). Other genes have been involved in intracellular trafficking and secretion (HAMP, NUP37, NUP98, RAB28, and SENP1). This approach showed that, in an HAM/TSP patient, a global activation of pathways involved in lymphocyte response to chemokine signal was associated with high CRMP2 expression.

We further focused on cultured T cell clones and evaluated the relationship between cell polarization, migratory rate, and CRMP2 expression. Polarization, the initial step of lymphocyte migration, was analyzed in 12 infected and 3 uninfected T cell clones by counting the cells exhibiting a bipolar shape (uropod formation). Lymphocytes of infected T cell clones displayed uropod at higher frequency (86 ± 6%) than uninfected clones (41 ± 7%) (p < 0.01). The migratory rate of T lymphocytes was specifically enhanced for infected T cell clones (Fig. 1C, left graph), and non-linear regression analysis of data using a logarithmic representation indicated a degree of correlation between the migratory rate and the frequency of CRMP2+ cells in each T cell clone (R² = 0.6647) (Fig. 1C, right graph). Blockade experiment using anti-CRMP2 Ab (Fig. 1D) showed the involvement of CRMP2 in the migration and polarization of infected T cell clones (Fig. 1D1, 1D2). In addition, treatment with CRMP2 siRNA reduced the number of migrating cells (Fig. 1D3). These data confirmed the role of CRMP2 in cell polarization status and migratory capacity of infected T cell clones. To conclude, HTLV-1 infection induced a profound alteration of the T cell motility machinery in parallel of modulation of CRMP2 expression.
The viral protein Tax is involved in the modulation of CRMP2 level and lymphocyte migratory rate

We suspected the virus protein Tax to regulate CRMP2 expression in infected T cell clones, because this protein is known to maintain chronic activation of T lymphocytes and also modulates expression of viral and cellular genes (11, 37). A set of experiments solidly supported this hypothesis. Analysis of Tax at mRNA level was performed by qPCR in virus-infected T cell clones (11 analyzed) (Fig. 2A). Linear regression analysis, which combined tax expression level and CRMP2+ cells frequency in each clone, revealed a degree of correlation between CRMP2 (positive cells in percentages) and tax expression level ($R^2 = 0.626$). Using RT-PCR and Western blotting, we also compared CRMP2 expression in chronically infected Tax-expressing T cell lines (C91PL, C8166/45, and HUT102 cell lines) and uninfected T cell lines (Jurkat and CEM) (Fig. 2B). Increased CRMP2 mRNA and protein levels in chronically infected T cells substantiated the association of Tax with elevated CRMP2 expression. However, Tax in T cell clones also reflected HTLV-1 proviral expression, suggesting that virus proteins other than Tax could modulate CRMP2 in infected lymphocytes. The role of Tax in modulation of CRMP2 expression level was confirmed in primary T lymphocytes transduced with HIV-based lentiviral vector (TRIP-Tax) to express Tax (Fig. 2C). TRIP-gfp was used as control (data not shown). Flow cytometry analysis detected large amount of Tax in transduced lymphocytes on day 2 (95% Tax+ cells) and elevated CRMP2+ cell frequency in parallel (77–90%). Likewise, Tax transfection in the T cell line Jurkat enhanced CRMP2 expression level, as shown by Western blotting performed at 24 and 48 h posttransfection (Fig. 2D). Thus, Tax protein per se was able to augment CRMP2 expression in T lymphocyte. Finally, to evidence a direct effect of Tax protein on T cell motility, TRIP-Tax–transduced T lymphocytes were examined for migratory property (Fig. 2E). Tax+ lymphocytes exhibited a higher migratory rate compared with lymphocyte control ($p = 0.01$). Altogether, these data implicated Tax in the motile phenotype displayed by HTLV-1–infected T lymphocytes via a modulation of CRMP2 expression level.

Virus infection affects CRMP2 phosphorylation and adhesion to cytoskeleton elements in T lymphocytes

In neural and immune cells, CRMP2 is known to mediate intracellular signaling of several membrane receptors, an activity regulated by the concerted action of diverse kinases, acting notably at the C terminus of the protein (reviewed in Refs. 25 and 38). In addition, CRMP2 activity also results from opposing activity of its two molecular subtypes, CRMP2-A and -2A/B, which occur from the alternative usage of coding exons. They indeed exert a contrastive effect on microtubule pattern and neuron morphology because CRMP2A supports axonal elongation by antagonizing to CRMP2A/B (39). We therefore investigated the consequence of virus infection on CRMP2 activity by analyzing CRMP2 subtypes...
and phosphorylated forms in chronically infected T cell lines. Western blotting was performed using polyclonal Abs directed against CRMP2-A, CRMP2-A/B (generally termed CRMP2), CRMP2-pSer522, CRMP2-pThr509, and CRMP2-pTyr479 (Fig. 3A). As expected, CRMP2-2(A/B) expression was elevated in virus-infected T cell lines. The most striking difference appeared in CRMP2-A expression, only detected in the uninfected cell line CEM. Interestingly, CRMP2-A is known for its blocking effect toward CRMP2(-A/B) in the control of neural cell polarity (39). In addition, CRMP2-pSer522, CRMP2-pThr509, and at a lesser extent, CRMP2-pTyr479, forms were enhanced in virus-infected cells, notably in productive infected cell lines, as shown by the ratio of phosphorylated forms to total CRMP2 (CRMP2-A/B) signal. Because Ser522, Thr509, and Tyr479 are target for Cdk5, GSK-3β, and the Src kinase Yes, respectively (25, 40), we analyzed the active form of these kinases using Western blotting (data not shown). None or few modifications were observed in infected versus uninfected T cell lines. The effect of Tax protein on CRMP2 phosphorylation was also investigated using tax transfection of Jurkat cells. Tax affected the level of CRMP2 as expected (1.6-fold increase), but only weakly changed CRMP2 phosphorylation (weak increase in pSer522 form) (Fig. 3B). To confirm this aspect, the infected T cell line HUT102 was transduced with lentivirus-expressing shRNA anti-Tax and vector control, as described previously (32), and cells were examined in Western blotting with anti-pCRMP2 Abs. Tax knockdown did not lead to significant modification of phosphorylation of CRMP2 on Ser522 and Tyr479, as shown by the ratio pCRMP2/CRMP2 (0.37 and 0.35, respectively). Overall, these data showed the ability of HTLV-1 infection to change the phosphorylation status of CRMP2, thus to modify its activity. However, Tax was not involved in this posttranslational modification.

In neural cells, phosphorylation modulates CRMP2 interaction with cytoskeleton elements in response to external signals, resulting in cytoskeleton reorganization, axonal elongation, and cell migration (41, 42). We then compared CRMP2-2(A/B) expression, only detected in the uninfected cell line CEM. Interestingly, CRMP2-A is known for its blocking effect toward CRMP2(-A/B) in the control of neural cell polarity (39). In addition, CRMP2-pSer522, CRMP2-pThr509, and at a lesser extent, CRMP2-pTyr479, forms were enhanced in virus-infected cells, notably in productive infected cell lines, as shown by the ratio of phosphorylated forms to total CRMP2 (CRMP2-A/B) signal. Because Ser522, Thr509, and Tyr479 are target for Cdk5, GSK-3β, and the Src kinase Yes, respectively (25, 40), we analyzed the active form of these kinases using Western blotting (data not shown). None or few modifications were observed in infected versus uninfected T cell lines. The effect of Tax protein on CRMP2 phosphorylation was also investigated using tax transfection of Jurkat cells. Tax affected the level of CRMP2 as expected (1.6-fold increase), but only weakly changed CRMP2 phosphorylation (weak increase in pSer522 form) (Fig. 3B). To confirm this aspect, the infected T cell line HUT102 was transduced with lentivirus-expressing shRNA anti-Tax and vector control, as described previously (32), and cells were examined in Western blotting with anti-pCRMP2 Abs. Tax knockdown did not lead to significant modification of phosphorylation of CRMP2 on Ser522 and Tyr479, as shown by the ratio pCRMP2/CRMP2 (0.37 and 0.35, respectively). Overall, these data showed the ability of HTLV-1 infection to change the phosphorylation status of CRMP2, thus to modify its activity. However, Tax was not involved in this posttranslational modification.

In neural cells, phosphorylation modulates CRMP2 interaction with cytoskeleton elements in response to external signals, resulting in cytoskeleton reorganization, axonal elongation, and cell migration (41, 42). We then compared CRMP2 subcellular localization in uninfected Jurkat and HTLV-1–infected C8166 T cell lines. Western blotting performed on membrane/endosome, nucleus, and cytoskeleton cell fractions showed, in C8166 cells, a higher recruitment of CRMP2 to the cytoskeleton compartment that paralleled its decrease in the membrane/endosomal fraction (Fig. 3C). Preferential CRMP2 adhesion to cytoskeleton in C8166 versus Jurkat T cells was also suggested by immunofluorescence analysis (Fig. 3D). Because CRMP2 is able to bind actin, tubulin, and the intermediate filament vimentin (23), we investigated whether virus infection modified CRMP2 binding to these proteins. Immunoprecipitation of tubulin, actin, and vimentin was performed on Jurkat and C8166 cells, and then, CRMP2 was evaluated in immunoprecipitates. It must be noted that CRMP2 is often expressed as a 62-kDa full-length and a 58-kDa short-processed form resulting from calpain-mediated cleavage (a 50-kDa product may appear). With Ab that recognizes the long and

### Table I. Gene specifically expressed or with elevated expression in blood CD69+ cells isolated from HAM/TSP patients

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<td>ARHGAP8</td>
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### Enhanced Expression (Top 10 Genes)

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<td>RGS16</td>
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short forms (CRMP2-pep4 Ab), Western blotting detected CRMP2 coimmunoprecipitation with tubulin, actin, and vimentin, as described previously. However CRMP2 pattern differed in C8166 versus Jurkat cells (Fig. 3). Notably, the full-length CRMP2 bound the three cytoskeleton components in C8166 cells but only vimentin in JK cells. To conclude, HTLV-1 modulated the phosphorylation status of CRMP2 and its recruitment to cytoskeleton elements in T lymphocyte.

**HTLV-1 acts on CRMP2 proteolytic processing, favoring the presence of active CRMP2 form in infected T lymphocytes**

We have recently reported the importance of proteolytic processing of CRMP2 in controlling the protein activity in neural cells (27). The short-processed CRMP2 form (58 kDa) antagonized the full-length (62 kDa), acting as a physiological dominant-negative signal to reduce neural cell process extension. This observation and the preferential adhesion of the full-length/active CRMP2 form to actin and tubulin in infected T cells prompted us to test the hypothesis that HTLV-1 enhanced CRMP2 activity in T lymphocytes by increasing the level of the CRMP2 active form. We further examined the presence of full-length and short CRMP2 in uninfected and HTLV-1–infected T cell lines using Western blotting and CRMP2-pep4 Ab (Fig. 4B). The short (58 kDa) and full-length (62 kDa) products were detected in all T cell lines, but the full-length CRMP2 was overexpressed in infected T cells, as shown by the 58:62-kDa ratio. The role of HTLV-1 in the modification of CRMP2 processing was then evaluated in tax-transfected Jurkat cells (Fig. 4C). Western blotting showed that CRMP2 level expression was enhanced in Tax expressing cells, as expected. In addition, the 58:62-kDa ratio was strongly reduced (51 to 9, in gfp- versus tax-transfected cells), indicating that Tax promoted the full-length active form in transfected lymphocytes. To confirm this aspect, infected T cell line HUT102 was transduced with lentivirus-expressing shRNA anti–Tax, and cells were examined for CRMP2 cleavage using Western blotting. Knockdown expression of Tax protein was associated with the reduction of full-length CRMP2, as shown by the augmentation of the 58:62-kDa ratio (3:21) in control lentivirus versus shRNA anti–Tax-treated cells).

As Tax is known to regulate the function of the proteasome, notably by binding specifically to two subunits of the 20S proteasome, HsN3 and HC9 (43), the evolving 58:62-kDa ratio of CRMP2 that we observed led us to analyze CRMP2 proteasomal...
degradation under Tax influence. Jurkat and C8166 cells were treated with the proteasome inhibitor PS-341 (10 nM for 24 h), allowing us to evaluate the fraction of CRMP2 protein subjects to degradation. The expression of the short and full-length forms of CRMP2 was analyzed by Western blot. We observed that the fold increase of short CRMP2 expression after PS-341 treatment was dramatically enhanced (108-fold more) in the infected cell line C8166 compared with the uninfected Jurkat one (Fig. 4D). By contrast, the expression of full-length CRMP2 was similarly affected in the two cell lines. These results suggest that HTLV-1 infection may contribute to the modification of the 58:62-kDa ratio by enhancing the degradation of the short form of CRMP2.

To substantiate a possible link between Tax, CRMP2 proteolytic processing and T lymphocyte migration, we examined the migratory rate of tax-transfected Jurkat cells following cotransfection with full-length or short CRMP2 (CRMP2wt and CRMP2Δ503). Tax enhanced the migratory rate of cotransfected Jurkat cells, as expected (Fig. 5A). In addition, in the presence of Tax, the cells transfected with CRMP2Δ503 showed no reduction of their migration ability compared with the control, contrary to what was observed with CRMP2Δ503 alone. Interestingly, Western blotting analysis of CRMP2 showed that transfection with CRMP2Δ503 was associated with a decreased detection of the endogenous full-length CRMP2 in Jurkat cells, whereas cotransfection with Tax restored this expression (Fig. 5B). All these data strongly suggested that the elevated migratory rate displayed by HTLV-1–infected T cell clones reported above was associated with high level of full-length/active CRMP2. This hypothesis was examined in eight infected T cell clones established from patients using CRMP-2-Cter and -pep4 Abs in Western blot analysis. Interestingly, the full-length CRMP2 was detected only in infected T cell clones (Fig. 5C). We then examined the possible association between the full-length form in T lymphocytes and their migratory rate (three infected and three uninfected T cell clones studied) following evaluation of the full-length/short (62:58 kDa) CRMP2 ratio (Fig. 5D, migration in lower panel, CRMP2 ratio in upper panel). We observed that the more favorable to the full-length form the ratio, the more elevated the migratory rate of infected T-cell clones was. Collectively, these data demonstrated that HTLV-1 infection, possibly through Tax activity, profoundly altered posttranslational processing of CRMP2, resulting in more active molecular form in T lymphocyte and elevated migration as a consequence.
Tax and CRMP2 colocalized at uropod and cell–cell contact in HTLV-1–infected T cells

Given 1) the crucial role of CRMP2 in remodeling neural cell and T lymphocyte cytoskeleton by direct binding to microtubules (25, 44) and also in participation, as a cargo receptor, in the transport of specific vesicles (45, 46), 2) the ability of Tax protein to modulate CRMP2 activity in infected lymphocyte, in particular cytoskeleton binding (shown in this paper), 3) the capacity of infected cell to transfer Tax though a well organized cell–cell contact (the so-called virological synapse (47), and 4) the role of microtubule in T cell signaling (48), we suspected CRMP2 to bind Tax protein at specific sites in lymphocyte cytoplasm to cooperate. Preliminary analyses using immunoprecipitation and CRMP2-GST pull-down strategy were unable to detect a direct association between these proteins. Nevertheless, we further analyzed the localization of these two proteins in infected lymphocyte. Immunofluorescence study showed the presence of Tax in lymphocyte cytoplasm, as small dots and its colocalization with CRMP2 at uropod in infected T cell clone and at the site of cell–cell contact in infected/productive HUT102 T cells (Fig. 6). This suggested a possible cooperation between these two proteins for uropod formation and subsequent lymphocyte migration and for molecule/signal delivery through membrane connection.

FIGURE 4. CRMP2 processing in HTLV-1–infected T cell lines and role of short CRMP2 in T lymphocyte migration. A, Migratory rate of JK cells transfected with full-length (CRMP2wt) and short (CRMP2-Δ503) CRMP2: elevation with CRMP2wt and reduction with the short/dominant-negative CRMP2-Δ503. B, Detection of the full-length/62kDa CRMP2 form (empty arrow) and cleaved/short product (58 kDa, black arrow) in T cell lines (western blot; anti-CRMP-2 pep4 Ab): full-length CRMP2 is elevated in infected cell lines, as shown by the decreased 58:62-kDa ratio. C, De novo Expression of Tax in JK cells (Tax plasmid versus empty vector) and extinction of Tax in infected HUT102 cells (lentiviral shRNA anti-Tax versus empty lentivirus): the 62-kDa form is elevated in tax transfected Jurkat cells and reduced following Tax knocking-down, as shown by the short/full-length CRMP2 ratio. D, Expression of CRMP2 full-length and short forms in Jurkat cells following treatment with proteasome inhibitor PS341 (fold increase): high increase in short CRMP2 expression (108-fold more) in C8166 cells compared with uninfected cells Jurkat. By contrast, full-length CRMP2 is similarly affected in the two cell lines.

FIGURE 5. Elevated expression of full-length CRMP2 in virus-infected T cell clones correlates their high migration competency. A, Migratory rate of Jurkat cells cotransfected with tax and CRMP2wt or CRMP2-Δ503: increased migratory rate in tax-transfected cells in all conditions. Tax abolished the negative effect of CRMP2-Δ503. B, Expression of endogenous CRMP2 in transfected Jurkat cells: Tax restores the full-length CRMP2 (62 kDa) expression decreased by CRMP2-Δ503. C, Patients’ T cell clone analysis using Western blotting: detection of full-length CRMP2 only in virus-infected T cell clones with anti–CRMP2-Cter Ab. D, Migration of uninfected and infected T cell clones (Transwell system): elevated migratory rate in virus-infected T cell clones is associated with higher level of full-length CRMP2 (62:58-kDa ratio).
infected T cell clones established from infected individuals, activates the lymphocyte locomotion machinery. Using virus-60–62). In this study, we describe a new strategy for HTLV-1 and molecule adhesion, as such was suspected to facilitate T lymphocytes express and can release the viral protein Tax, a strong inducer of proinflammatory cytokine secretion from the T lymphocyte thus to positively control the migratory activity of gene expression profile in activated lymphocytes of HAM/TSP patients revealed the activation of T cell locomotion machinery. Altogether, this suggests that HTLV-1 infection chronically activates the molecular pathways involved in lymphocyte motility, notably those linked to CRMP2. Several observations support this hypothesis. First, CRMP2 belongs to the PI3K/Akt/GSK-3 pathway, shown essential for axon elongation in neurons (41) and for cell migration in general (72). The ability of HTLV-1 to activate PI3K/Akt signaling pathway in T lymphocyte has been demonstrated (73) and could explain the specific gene profile found in cells selected from HAM/TSP patients that involves the molecular G protein/PI3K pathway. Interestingly, CRMP2 is a binding partner of several proteins of this pathway, including Fyn, PI3KR1/p85, and Vav-1 (25, 42). Finally, Tax may bind PI3KR1/p85 (37) and influence Vav phosphorylation (74). To conclude, HTLV-1 infection has the ability to influence the CRMP2/PI3K/Akt/GSK-3 axis in T lymphocyte thus to positively control the migratory activity of infected cells. The present observation on ex vivo lymphocytes complements two previous reports showing that HTLV-1 regulates G protein signaling, in particular, activates the SDF1/CXCR4 axis in T lymphocyte of ATL patients, lymphoblastoid cells from Tax transgenic mice, and HTLV-1–infected T cell lines (75, 76).

CRMP2 proteolytic cleavage is an additional posttranslational modification essential for cell motility/outgrowth. In fact, CRMP2 is expressed in neural cells as a full-length (62 kDa) and a short-processed form (58 kDa), resulting from cleavage by the calcium-dependent protease calpain (77). CRMP2 cleavage is enhanced in injured neural tissues displaying limited postinjury remodeling and neurite regeneration (78). Our demonstration that the short CRMP2 molecular form negatively regulates axon elongation could explain in part this feature (29). In the context of immune cells, we have recently identified the full-length and short CRMP2 forms in T lymphocytes (25) and showed, in the present work, that the negative regulation of short CRMP2 (CRMP2Δ503) occurs in lymphocyte migration. Interestingly, cotransfection experiments pointed out the ability of Tax protein to overcome the negative effect of short CRMP2 on T cell migration. Such a feature could have an explanation at protein level as transfection with CRMP2Δ503 dramatically reduced the full-length CRMP2 in Jurkat cells, whereas cotransfection with Tax restored this expression. Given these data, privileging the full-length/active CRMP2 form in T lymphocyte...
could be a means for HTLV-1 to promote cell migration. The following observations support this hypothesis: 1) the ratio of CRMP2 forms was always more in favor of the full-length protein in infected T cell clones, tax-transfected T cells, and infected T cell lines; 2) knocking down Tax expression through lentiviral shRNA strategy resulted in decreased level of the full-length protein in infected lymphocyte; 3) the full-length CRMP2 form, known as a direct modulator of microtubules (44), was bound to the microtubule protein tubulin in HTLV-1–infected T cell line and not in control T cells; and 4) the presence of full-length CRMP2 in infected T cell clones correlated with elevated migratory rate. The high level of full-length CRMP2 in infected T lymphocytes could result either of a less effective protein cleavage or a modified stability of one or another form of CRMP2. We observed that HTLV-1 infection could result in an important increase of the proteasomal degradation of the short CRMP2, supporting the modifications of the 58:62-kDa ratio observed. Although the precise mechanism of CRMP2 modulation remains unclear, it obviously appears that HTLV-1 exploits a fundamental cell regulatory mechanism to enhance lymphocyte motility.

The viral protein Tax appeared to be involved in the modulation of CRMP2 level and cleavage but not in the enhanced phosphorylation displayed by HTLV-1–infected lymphocytes, suggesting that other viral proteins can modify CRMP2 expression and activity. Nevertheless, colocalization of Tax and CRMP2 at cell sites important for cell migration (uropod) and molecule/signal delivery (membrane cell–cell contact) strongly suggests the cooperation of these two molecules to support functions that are important for HTLV-1 virus/protein transmission (79, 80). This interesting aspect of CRMP2 function in virus-infected lymphocyte remains to be analyzed.

Other neuroinflammatory/neurodegenerative diseases share the importance of infiltrating T lymphocytes in pathogenesis. A link between diffuse axonal loss, a key feature of multiple sclerosis, and the presence of meningeal T lymphocytes in spinal cord of patients was reported recently (81). In addition, recent studies assigned an unexpected role to infiltrated T lymphocytes in the pathophysiology of Parkinson’s disease and Amyotrophic lateral sclerosis (82, 83). Chronic activation of cell signaling programs that directs immune cell motility and includes CRMP2 could be a common mechanism leading to CNS infiltration and tissue damage in various neuroinflammatory diseases. This point merits to be investigated.

To conclude, our findings provide an additional clue to understanding the migration and infiltration of HTLV-1–infected lymphocytes into various tissues and suggest that the regulation of CRMP2 activity by retrovirus infection is a novel aspect of virus-induced neuroinflammation.

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