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Measles Virus Interacts with and Alters Signal Transduction in T-Cell Lipid Rafts

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By a contact-dependent surface interaction, the measles virus (MV) glycoprotein complex induces a pronounced inhibition of T-cell proliferation. We now show that MV directly interacts with glycosphingolipid-enriched membrane microdomains on human primary T cells and alters recruitment and segregation of membrane proximal signaling components. Contact-dependent interference with T-cell receptor-stimulated tyrosine phosphorylation and Ca mobilization is a late event seen 24 h after MV treatment. In contrast, stimulated recruitment of pleckstrin homology domain-containing proteins such as Akt and Vav is inhibited early after MV contact, as is segregation of the activated Akt kinase from rafts. Tyrosine phosphorylation of the regulatory subunit of the phosphatidylinositol 3-kinase (PI3K), p85, is apparently normal then, yet this protein fails to partition to the lipid raft fraction, and this is associated with stable expression of its negative regulator Cbl-b. Thus, by interaction with lipid rafts, MV contact initially targets recruitment of PI3K by preventing stimulated Cbl-b degradation and activation of PI3K-dependent signaling components.

T-cell signaling after antigen contact requires spatial recruitment and temporally regulated activation of components of the signalosome (1). For this to occur, glycosphingolipid-cholesterol enriched membrane microdomains, also referred to as lipid rafts, are essential. They provide platforms involved in this process as they concentrate and segregate proteins and phospholipids essentially required for signal initiation and propagation (21). Early membrane-proximal signaling requires activation of the Src kinases (Lck and FynT) which phosphorylate substrates such as the ITAMs within the T-cell receptor (TCR) complex. After recruitment and activation, Zap-70 phosphorylates downstream substrates, including the raft resident LAT adaptor protein, which then provides docking sites for the formation of signaling complexes. LAT links TCR signaling to phospholipase C-γ activation, Ca mobilization, and subsequent activation of mitogen-activated protein kinases and NFAT, providing the first signal for T-cell activation. For optimal induction of cytokine secretion, proliferation, and cell survival, a second, costimulatory signal is provided via CD28, which initiates, via PKCθ, the NF-κB pathway (16, 18) and also the phosphatidylinositol 3-kinase (PI3K) pathway (27). It has been shown that the regulatory subunit of this kinase, p85, is recruited to tyrosine residue 173 within the cytoplasmic tail of human CD28 and that this is important for CD3/CD28-dependent activation of mature T cells (28). CD28 ligation was found to target Cbl-b, an E3 ubiquitin ligase, for ubiquitination and proteasomal degradation, thereby relieving Cbl-b-interacting proteins including p85 for activation-induced recruitment to CD28 (11, 12, 22, 25, 41). By the activity of the membrane-recruited PI3K, phosphoinositides which are enriched within the lipid rafts are phosphorylated at the D3 position of the inositol ring to yield phosphatidylinositol 3,4-biphosphates (PIP_2) and phosphatidylinositol 3,4,5-triphosphates (PIP_3) (2, 15). These serve as second messengers recruiting pleckstrin homology (PH)-containing proteins such as the guanosine exchange factor Vav and the Akt kinase to the membrane, where they are further activated by phosphorylation (6). The absolute requirement of antigen-triggered T-cell proliferation for sustained PI3K activity and transport of PH domain-containing proteins to the immunological synapse has been directly demonstrated (7, 14).

The inability of lymphocytes isolated from patients with acute measles to proliferate in response to mitogenic and TCR stimulation ex vivo is a hallmark of measles virus (MV)-induced immunosuppression. In vitro evidence suggests that MV infection interferes with the viability, maturation, and function of professional antigen-presenting cells, which may promote T-cell apoptosis or suppression of cellular immunity by an imbalanced cytokine release (31). It has, however, also been described that the MV glycoprotein (gp) complex consisting of the hemagglutinin (H) and fusion (F) proteins expressed on infected cells or UV-inactivated virions induces a state of proliferative unresponsiveness in uninfected T cells by surface contact (8, 32, 38). Characteristically, primary T cells contacted by the MV gp complex are refractory to mitogen-, allogen-, and CD3/CD28-induced proliferation (29). In addition, proliferation of T-cell lines such as Jurkat and interleukin-2 (IL-2)-dependent Kit-225 cells is also efficiently blocked in a gp dose-dependent manner (4). As T cells do not undergo apoptosis in this system and upregulation of early activation markers is unaffected, gp-dependent signaling apparently specifically targets signaling pathways promoting S-phase entry of T cells (10, 33).

We have previously shown that the IL-2-dependent Ser473 phosphorylation and activation of the Akt kinase, but not the JAK/STAT pathway, is blocked within a few hours of MV treatment in IL-2-dependent Kit-225 and primary T cells and that this was important for MV-induced proliferative unresponsiveness (4). Since Akt fused to a myristoylation sequence, when overexpressed in Jurkat T cells or spleen cells from transgenic mice, largely compensates for negative signaling by

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MV, membrane recruitment of this kinase is likely targeted. We now show that MV directly interacts with T-cell lipid rafts, thereby causing profound alterations in their ability to recruit and segregate proteins central to TCR activation, such as the activated Akt kinase and Vav. As another MV-induced early event, CD28-dependent proteasomal degradation of Cbl-b is prevented. Although TCR-stimulated Ca mobilization and tyrosine phosphorylation of signaling components including p85 are unaffected after short MV contact, p85 fails to associate with lipid rafts, providing a likely explanation for inefficient generation of PIP_3 and the subsequent failure of PH domain-containing proteins to be recruited.

MATERIALS AND METHODS

Cells and viruses. Primary human T cells were isolated from sheep erythrocyte complexes by erythrocyte lysis or were enriched from peripheral blood lymphocytes by use of nylon wool columns. Jurkat T cells were kept in RPMI 1640 plus 10% fetal calf serum (FCS). MV wild-type strain WTF (grown on lymphoblastoid BJAB cells in RPMI 1640–10% FCS) and vaccine strain Edmonston (ED) (grown on Vero cells in minimal essential medium–5% FCS) were titrated on marmoset lymphoblastoid B95a cells (kept in RPMI 1640–10%FCS). Viruses were UV inactivated (1.5 J/cm^2) and, when indicated, purified by gradient centrifugation or adjusted for equal amounts of gp content after Western blot analysis. Mock preparations were obtained from uninfected BJAB or Vero cells. For binding assays, T cells were cocultured with virus or mock preparation for 2 h at 4°C, followed directly by CD3/CD28 stimulation at 37°C. Upon exposure to MV at 37°C, T cells were kept in RPMI 1640–10% FCS supplemented with 0.2 mM fusion-inhibiting peptide (Z-D-Phe–L-Phe-Gly-OH; Bachem). Under these conditions, infection of T cells throughout the experiment was completely abolished as controlled for by staining for intracellular MV (not shown). UV-inactivated viruses were used at amounts corresponding to a live virus multiplicity of infection of 0.5.

Antibodies and immunoprecipitation. Akt (C-20)-, Vav (C14)-, pTyr (PY99)-, hemagglutinin (HA) tag (F-7)-, Cbl-b (G-1 or H-121)-, and LAT (FL-233)-specific antibodies were obtained from Santa Cruz Biotechnology; PI3K-specific antibody (rabbit antiserum) was obtained from Upstate, and phospho-Akt (Ser473)-specific antibody was obtained from BioLabs. Anti-mouse–Fluor 488 and cholera toxin (CTx)–Alexa 594 (Molecular Probes) were used for fluorescence analysis. Monoclonal anti-human CD3 (clone UCHT1) and anti-CD28 (clone ANC28.1/5D10) antibodies were purchased from Ancell. For cross-linking experiments, a goat anti-mouse antibody (Dianova, Hamburg, Germany) was used. The monoclonal anti-MV-H antibody (K4) was produced in our laboratory and conjugated with Alexa 488 fluorophor according to the manufacturer's procedure. The moesin-specific antibody was kindly provided by J. Schneider-Schaulies, Institute for Virology and Immunobiology, Würzburg, Germany. Immunoprecipitation was performed by standard procedures with the HA antibody for 2 h at 4°C.

T-cell stimulation and raft fractionation. When indicated, 1×10^8 primary T cells or 5×10^7 Jurkat T cells were stimulated in 200 μl of Hanks balanced salt solution (containing 1 mM HEPES, pH 7.5) at 37°C for 30 min with 4 μg of anti-CD3 and anti-CD28 premixed with 4 μg of goat anti-mouse immunoglobulin. Cold Brij 98 lysis buffer (0.1% Brij 98 in NTE buffer [25 mM Tris {pH 7.5}, 150 mM NaCl, 5 mM EDTA, 1 mM Pefabloc, 1 mM Na_3VO_4, 1 mM NaF]) was added at 0°C and left for 20 min. After being mixed with an equal volume of 80% sucrose in NTE buffer, the lysate was overlaid with 2 volumes of 30% sucrose in NTE buffer followed by a 50% volume of NTE buffer in a tube and centrifuged at 200,000 × *g* for 22 h at 4°C. Fractions were harvested (bottom to top) and precipitated with 2 volumes of cold acetone. Samples were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and analyzed by immunoblotting.

Analysis of intracellular calcium concentration levels by flow cytometry. Primary T cells (10^6) were washed once in Hanks balanced salt solution (without $CaCl_2$, $MgSO_4$, and phenol red) containing 5% FCS and 25 mM HEPES (pH 7.5). Cells were loaded with 1 μM Fluo-4 as cell-permanent AM ester (Molecular Probes) at 37°C for 30 min, washed, and reincubated at37°C for 30 min in Ca indicator-free medium. Finally, complete Hanks medium was added and Ca flux was determined by with a FACSCALIBUR (Becton Dickinson). After 20 s of acquisition, anti-CD3 antibody (20 μg/ml) was added, and acquisition was continued for a total of 200 s. Ionomycin (10 μM) stimulation was performed to evaluate cell loading with the Fluo-4 indicator. Analysis of data was done by using the Flow Jo (Tree Star, Inc.) software program.

Transfections. Primary T cells (10^7) were transfected with 10 μg of pGFP-Akt PH (kindly provided by A. Gray, University of Dundee, Scotland, United Kingdom) (13) or pCG-p85αHA (kindly provided by A. Carrera, Department of Immunology and Oncology, Madrid, Spain) (17) with a human T-cell nucleofector kit (Amaxa, Cologne, Germany) according to the manufacturer's instructions and were analyzed or used for further experimentation 20 h after nucleofection. For quantitative fluorescence analysis, the percentage of 100 to 200 T cells translocating the green fluorescent protein (GFP)–Akt-PH fusion protein after CD3/CD28 stimulation was determined.

Fluorescence analysis. Lipid rafts were stained with CTx conjugated with Alexa 594 (Molecular Probes) at 4°C and patched by addition of rabbit anti-CTx (Sigma-Aldrich) at 37°C for 20 min. T cells were plated onto poly-L-lysine-coated coverslips and fixed directly after lipid raft patching. MV colocalization with lipid patches was detected by staining with the Alexa 488-conjugated anti-H monoclonal antibody K4. Antigen loading of dendritic cells (DCs) and T-cell stimulation were performed as described previously (14). Briefly, DCs were differentiated from monocytes in the presence of 500 U of granulocyte-macrophage colony-stimulating factor per ml and 250 U of IL-4 per ml for 7 days, followed by lipopolysaccharide treatment for 24 h. DCs (5×10^4) were plated on glass coverslips coated with a 1:50 dilution of polylysine (Sigma-Aldrich) for 20 min at 4°C and incubated with 0.1 μg of *Staphylococcus* enterotoxin B (Sigma-Aldrich) per ml for 20 min at 37°C prior to addition of 10^5 transfected T cells and incubation for a further 20 min at 37°C. Conjugates were fixed in 4% paraformaldehyde and used for fluorescence analysis. For intracellular staining, the p-Tyr-specific antibody PY99 (Santa Cruz Biotechnology) was used. Cells were analyzed by microscopy, and for quantitative analysis, the percentage of 100 to 200 cells accumulating p-Tyr at the DC–T-cell contact site was determined.

RESULTS

MV interacts with lipid raft membrane microdomains on T cells. Signals elicited by TCR stimulation require a spatially and temporally regulated recruitment of molecules into or segregation of molecules from membrane microdomains referred to as lipid rafts. To study whether MV can interact with lipid rafts, T cells were left unstimulated and incubated with UV-inactivated MV (wild-type strain WTF) at 4°C to allow binding. As revealed by subsequent staining, MV (detected by an H protein-specific antibody) perfectly colocalized with patches obtained by antibody cross-linking of CTx, which interacts with GM1 (a raft marker glycolipid) (Fig. 1A). With the ED vaccine strain of MV, binding to but also outside the lipid rafts was observed (not shown); the latter was most likely due to the interaction of this strain with CD46, a nonraft resident molecule present also on resting primary T cells (Fig. 1B, left panel). CD150, the receptor for all MV strains, including WTF, on lymphocytes is not expressed in resting T cells (not shown). WTF binding was largely abolished in methyl-β-cyclodextrin-treated T cells (not shown), confirming that neither CD46 nor CD150 is responsible for MV-lipid raft interaction. We were also able to show the interaction of MV WTF with lipid rafts biochemically, since MV (detected by the MV gp H-specific antibody) almost completely cosedimented with the nonsoluble, LAT-containing membrane fraction (Fig. 1C, middle panel). When MV alone was applied to the sucrose gradient in the absence of cell membranes, only a minor proportion of the H protein was detected in the upper sucrose gradient fractions (Fig. 1C, upper panel). This indicates that the MV requires T-cell interaction to sediment into this particular fraction of the gradient (Fig. 1C).

Overall tyrosine phosphorylation in T cells is affected late after MV contact. To assess the consequences of MV interaction with early events in TCR signaling, overall tyrosine phos-

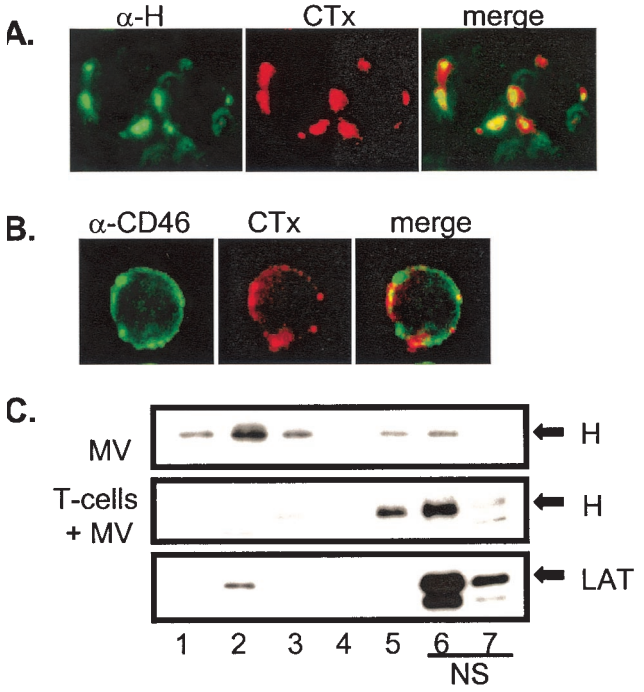

FIG. 1. MV binds to lipid rafts on the T-cell membrane. (A) Primary T cells were cocultivated with MV strain WTF (at 4°C for 2 h), followed by GM1 staining with CTx-Alexa 594 conjugate and patching with anti-CTx serum at 37°C. After fixation, cells were stained with an MV-H-specific antibody (α-H) and an Alexa 488-conjugated secondary antibody. (B) Lipid raft staining and patching was done as described for panel A, and CD46 was detected by using a specific monoclonal antibody and Alexa 488-conjugated secondary antibody. (C) Extracts prepared from WTF alone (upper panel) or T cells cocultured with WTF (2 h, 4°C) were subjected to sucrose gradient centrifugation and analyzed for the partitioning of LAT (as a raft marker) (lower panel) or for the MV H protein (upper and middle panels) by Western blot analysis. NS, nonsoluble fraction at the top of the sucrose gradient.

phorylation levels after TCR triggering were analyzed in T cells exposed to MV WTF for various time intervals. Under binding conditions (2 h at 4°C), tyrosine phosphorylation of proteins in both the detergent-soluble and -insoluble fractions did not differ between WTF- and mock-treated cells (Fig. 2A, left panel). Accumulation of tyrosine-phosphorylated proteins could also be directly visualized in MV-treated T cells at the contact zone with SEB-pulsed DCs (Fig. 2B, left lower panels and right panel). Despite still interacting with these cells, accumulation of tyrosine-phosphorylated proteins in T cells at the contact zone was strongly reduced 24 h after the initial MV interaction (Fig. 2B, middle panels and right panel). Similarly, tyrosine-phosphorylated proteins largely failed to partition to lipid rafts in T cells 24 h after MV treatment (Fig. 2A, right panel). In agreement with these observations, anti-CD3-stimulated Ca mobilization in primary T cells was found normal at 2 h and completely abolished at 24 h after MV treatment (Fig. 2C).

MV contact interferes with anti-CD3/CD28-induced Akt kinase recruitment to and segregation from lipid rafts in T cells. Our previous studies revealed that the IL-2-dependent activation of the Akt kinase in T cells is severely inhibited within a few hours after MV contact and that this is essential for inhibition of stimulated T-cell expansion. As T cells expressing Akt fused to a myristoylation sequence were previously found to largely resist MV negative signaling (4), we reasoned that TCR-stimulated membrane recruitment of the Akt kinase might be affected and that this, in contrast to the impact on tyrosine phosphorylation, might be an early event. To study Akt membrane recruitment in primary T cells, these cells were nucleofected with a construct encoding the GFP-tagged Akt PH domain and then CD3/CD28 stimulated (Fig. 3A and B) or cocultured with SEB-pulsed DCs (Fig. 3C and D). Pretreatment with mock preparations did not interfere with membrane recruitment of the tagged PH domain (Fig. 3A, left panels, and C, lower left panel), while this was essentially abolished in T cells exposed to MV (both ED and WTF) for 2 h (Fig. 3A and C, second and third lower panels, and B and D), as seen in control cells treated with wortmannin (Fig. 3A and C, fourth lower panels, and B and D).

Upon CD3/CD28 activation of primary T cells, the Akt kinase was efficiently recruited to lipid rafts (Fig. 4A). Pretreatment with mock supernatant also allowed for CD3/CD28-stimulated raft recruitment of a significant fraction of the Akt kinase, while this was almost completely abolished in primary T cells exposed to WTF (and also ED [not shown]) 2 h prior to stimulation (Fig. 4B, upper panel). Since membrane recruitment of the tagged Akt PH domain was affected by MV treatment (Fig. 3), this might also be the case for other PH domain-containing proteins. Indeed, Vav was also inefficiently relocated to the membrane in CD3/CD28-activated T cells exposed to UV-inactivated MV (Fig. 4C). To investigate whether segregation of signaling components from lipid rafts, which is also essential for T-cell activation, would also be affected by MV, we analyzed Jurkat T cells. There, the association of the activated Akt kinase in the raft fraction is prolonged in the absence of stimulation due to the absence of the lipid phosphatases PTEN and SHIP. The majority of Akt kinase protein, however, segregated from lipid rafts 30 min after TCR stimulation (Fig. 4D, upper panel). This was also observed in mock-treated cells, while a significant proportion of Akt was retained in the raft fraction in UV-inactivated MV-treated Jurkat cells (Fig. 4D, lower panel). Together, these data indicate that recruitment of PH domain-containing proteins such as Akt kinase and Vav to the lipid rafts is affected early after MV contact. The fact that Akt protein segregation from rafts is delayed in Jurkat cells, moreover, suggests that MV profoundly alters protein movement in lipid rafts.

Lipid raft recruitment of the PI3K regulatory subunit, but not its tyrosine phosphorylation, is affected early after MV contact. The lack of membrane recruitment of PH domain-containing proteins implies that accumulation of the PIP_3 pool in the membrane is reduced early after MV contact, and this could involve interference with the activation of PI3K. As the activity and membrane transport of this kinase depend on its regulatory subunit, we analyzed tyrosine phosphorylation levels and raft recruitment of p85. As the levels of this protein are low and only a fraction of this protein is tyrosine phosphorylated upon stimulation in primary T cells, these cells were transfected to overexpress an HA-tagged p85 (HA-p85). In agreement with our findings for overall tyrosine phosphorylation levels (Fig. 2), CD3/CD28 stimulation-dependent tyrosine phosphorylation of p85 did occur at 2 h following MV treatment (Fig. 5A, lane 2), while in T cells at 24 or 48 h after MV

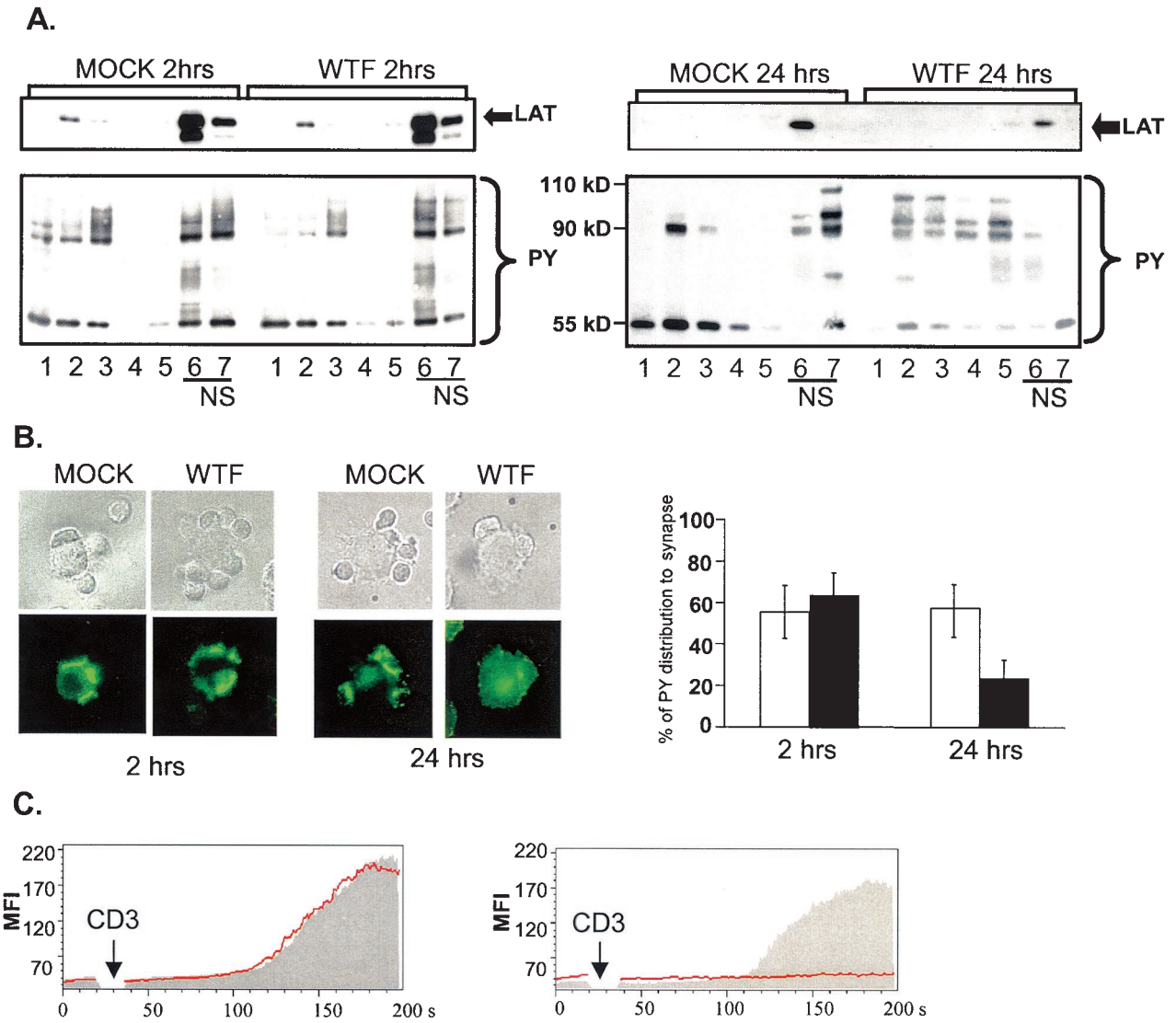

FIG. 2. MV affects stimulation-induced tyrosine phosphorylation in T cells at 24 h but not 2 h after exposure. (A) Extracts of primary T cells cocultivated with UV-inactivated MV WTF (or equivalent amounts of mock preparations) for 2 h at 4°C (left panels) or for 24 h at 37°C (right panels), followed by CD3/CD28 stimulation for 30 min, were analyzed for partitioning of LAT (NS, nonsoluble top fractions 6 and 7) (upper panels) or tyrosine-phosphorylated proteins (lower panels). (B) T cells treated with UV-inactivated WTF (or the corresponding amounts of the mock preparation) for 2 h at 4°C (left four panels) or for 24 h at 37°C (middle four panels) were cocultured with SEB-pulsed DCs for 20 min at 37°C, fixed, and stained with a phosphotyrosine-specific antibody and subsequently with an Alexa 488-conjugated secondary antibody. To visualize phosphotyrosine in T cells exposed to MV for 24 h, fluorescence was intensified. For quantification, the percentage of T cells translocating phosphotyrosine to the cortical membrane at the DC/T-cell interface was determined (right panel). Error bars indicate standard deviations. (C) The CD3-stimulated Ca^{2+} flux in T cells exposed to UV-inactivated WTF (red lines) for 2 h at 4°C (left panel) or for 24 h at 37°C (right panel) was determined. For all panels, the results of one representative experiment out of three independent experiments are shown.

interaction, tyrosine phosphorylation was almost completely abolished (Fig. 5A, lanes 5 and 7). Although at 2 h following MV treatment p85 was tyrosine phosphorylated, the regulatory subunit of the PI3K failed to associate with the lipid raft fraction in T cells exposed to MV (Fig. 5B), indicating that intracellular trafficking of this protein is impaired by MV contact. Cbl-b acts as a negative regulator of PI3K, and CD28-dependent degradation of Cbl-b is important for p85 recruitment. When analyzing Cbl-b levels after CD3/CD28 stimulation, we found that this protein is efficiently degraded in mock-treated T cells (although with slight donor-dependent differences in kinetics) but not in those exposed to MV for 2 h (Fig. 5C). Thus, MV contact apparently interferes with TCR-induced proteasomal degradation of the negative regulatory protein Cbl-b, and this might explain the lack of raft recruitment of p85 and thereby the inhibition of PI3K activation.

DISCUSSION

The inability of peripheral T cells to expand in response to polyclonal and TCR stimulation ex vivo is a hallmark of MV-induced immunosuppression, yet the molecular mechanisms

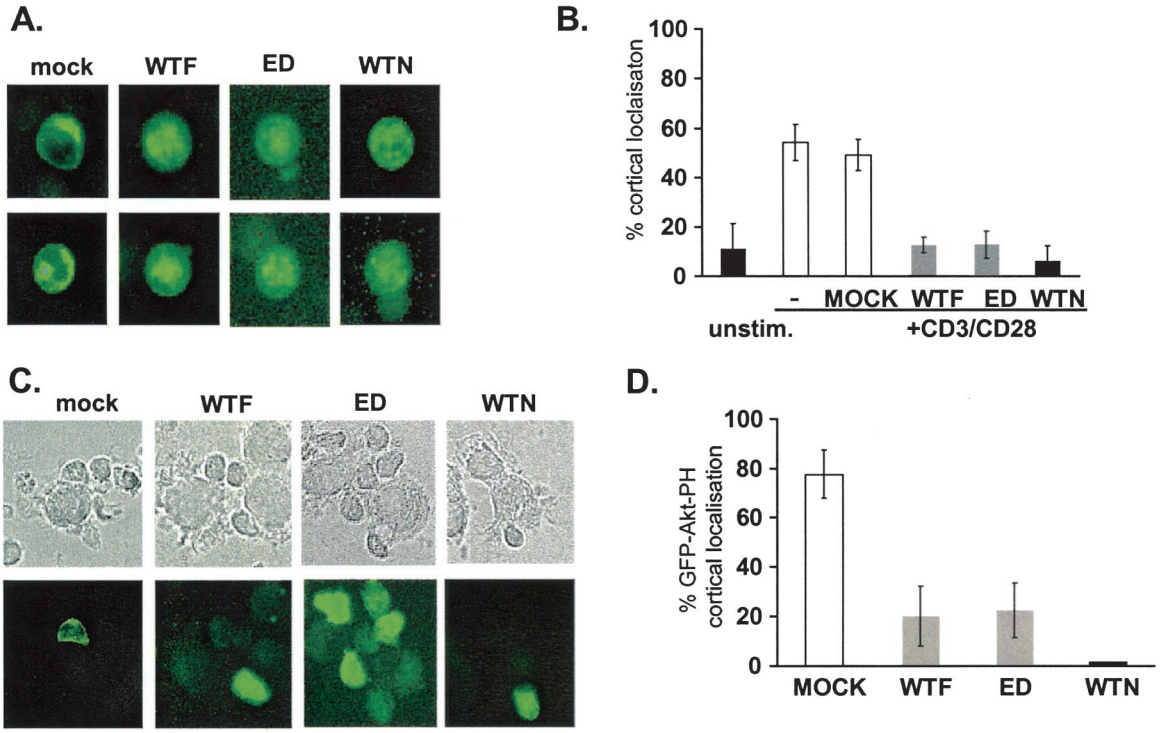

FIG. 3. UV-WTF interaction prevents stimulated membrane accumulation of the Akt-PH domain. (A and C) Primary T cells nucleofected with a GFP–Akt-PH domain construct were pretreated with mock extracts, UV-inactivated WTF or ED (2 h, 4°C), or wortmannin (20 min) prior to CD3/CD28 stimulation (20 min) (A) or incubation with SEB-pulsed DCs (20 min, 37°C) (C) (two examples for each are shown). (B) The percentage of T cells translocating the GFP–Akt-PH domain to the cortical membrane after treatment with medium (unstim.) or with CD3/CD28 alone, with mock treatment, or in the presence of UV-inactivated WTF, UV-inactivated ED, or wortmannin (WTN) was determined. (D) Percentage of mock-, UV-inactivated WTF-, UV-inactivated ED-, or wortmannin-treated T cells translocating the GFP–Akt-PH domain to the cortical membrane at the DC/T-cell contact zone. For panels B and D, standard deviations were calculated from five different fluorescence images, each containing 20 to 50 GFP-positive cells.

underlying this phenomenon are still ill defined. We now show that MV directly interacts with lipid rafts and that this leads to profound alterations of TCR-stimulated dynamics of signaling proteins within lipid rafts and deregulation of their activity. The T cells used in this study were purified by rosetting with sheep erythrocytes, which relies on their binding to CD2, or on nylon wool columns. This, however, did not have any detectable impact on subsequent TCR stimulation, since the results obtained with the mock control are in full agreement with the published literature. Moreover, identical results were obtained when peripheral blood lymphocytes were used (not shown).

Interactions of viruses with lipid rafts have gained much attention in regard to virus binding, in regard to activation of signaling pathways by viruses, and, finally, as important platforms for viral assembly and budding. There is increasing support for a role of lipid rafts in assembly and exit of viruses, including MV (5, 23, 24, 35, 37), and for interactions with these structures during receptor-mediated endocytosis or transcytosis (5, 26). We now show that MV also interacts with lipid rafts on resting T cells (Fig. 1A and C) and that this, however, occurs independently of its known binding receptors. CD150, the receptor for both ED and wild-type strains such as WTF (36), is expressed only after activation of T cells (34). CD46 is not a raft resident protein (24) (Fig. 1B) and interacts exclusively with attenuated strains such as ED. Interestingly, c-Cbl (which is $p120^{CBL}$ rather than the Cbl-b investigated by us [Fig. 5C]) and LAT were found to be tyrosine phosphorylated upon antibody ligation of CD46 in peripheral blood lymphocytes and were found to promote rather then inhibit proliferation of human T cells (3). Indeed, CD46, albeit at high concentrations, can provide a costimulatory signal in CD3-activated T cells as reflected by an increase in Vav tyrosine phosphorylation and membrane recruitment and activation of Rac1, but not cdc42 and Rho (40). Whether this also applies for CD46 cross-linking by MV has not been addressed in this study. Since early after MV contact the overall levels of tyrosine phosphorylation were found to be unaffected (Fig. 2A), it is likely that this also applies to Vav. It is, however, essentially clear that, in contrast to the results of study cited above, TCR-stimulated membrane recruitment of this protein is prevented in our system (Fig. 2B and C). Our data thus imply that the MV gp complex interacts with lipid raft components. MV interaction with membrane lipids cannot be excluded. The cell type specificity of MV negative signaling seems, however, to argue against a direct interaction of the MV gp complex with membrane lipids. Proliferative inhibition elicited by this complex is seen only in cells of hematopoetic origin (29), as is inhibition of stimulated Akt kinase activation (4). Although this has not yet been formally proven, it is likely that the effector domain resides within the proteolytically activated $F_{1/2}$ heterodimer (38, 39), as also ev-

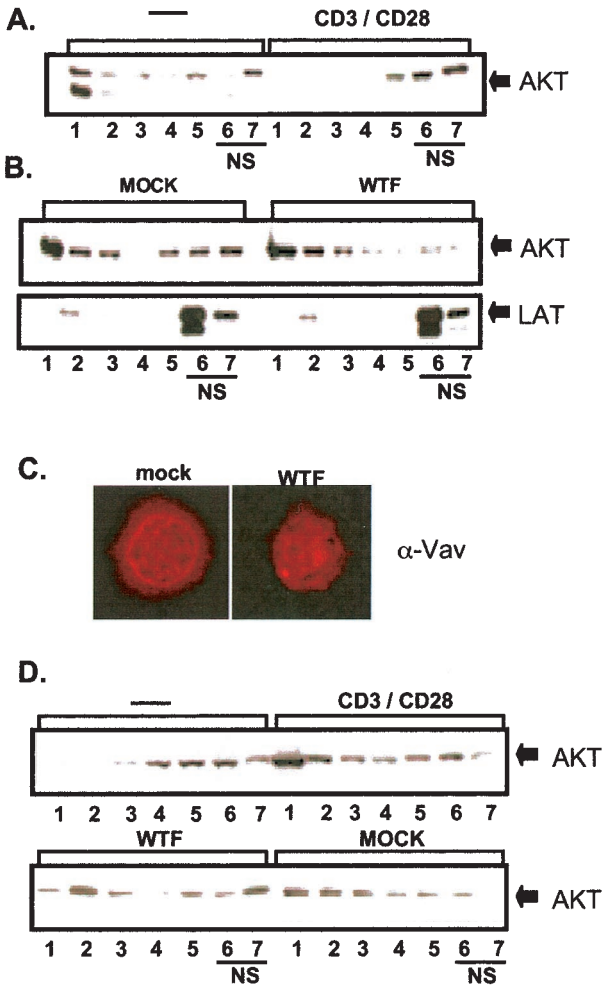

FIG. 4. MV interferes with CD3/CD28-stimulated membrane recruitment of Akt and Vav proteins. (A) Lysates prepared from primary unstimulated (left lanes 1 to 7) or CD3/CD28-stimulated (right lanes 1 to 7) T cells were used to detect Akt protein. NS, nonsoluble. (B) Primary T cells were mock or MV (WTF strain) treated for 2 h at 4°C and subsequently CD3/CD28 stimulated for 30 min. Cell lysis, raft isolations, and anti-Akt and anti-LAT (raft marker) immunoblottings were performed. (C) T cells mock pretreated or pretreated with WTF for 2 h at 4°C were CD3/CD28 activated and stained for Vav protein. (D) Jurkat T cells were serum starved and left unstimulated (upper panel, left lanes 1 to 7) or stimulated with anti-CD3/CD28 for 30 min alone (upper panel, right lanes 1 to 7) or after pretreatment with mock extract or WTF (lower panel) for 2 h at 4°C. Lysate fractionation and anti-Akt immunoblotting were performed as described for panel A.

idenced for the related respiratory syncytial virus (30). Recently, a novel receptor for the MV N protein has been suggested to be involved in immunosuppression (20). Since this as-yet-unidentified protein is, however, not expressed on resting T cells, it is unlikely to be involved in our system.

Indicating that MV contact does not affect TCR expression, TCR-stimulated tyrosine phosphorylation levels of proteins within and outside the rafts are apparently normal early after MV contact, as is Ca mobilization (Fig. 2). Our previous study and this study revealed interference with IL-2 receptor- and TCR-dependent activation and raft recruitment of the Akt kinase and probably other PH domain-containing proteins by MV contact (4) (Fig. 3 and 4A and B). As additional evidence that MV binding directly affects membrane protein dynamics, segregation of the Akt kinase from lipid rafts is delayed in Jurkat T cells (Fig. 4D). This could be addressed only in Jurkat cells, where the duration of Akt association with these microdomains is prolonged due to the absence of the PTEN phosphatase. These findings indicate that even if a minor fraction of Akt possibly still partitioned into the raft fraction, this would be unable to activate its downstream substrates efficiently.

We observed previously that Akt kinase activation after IL-2 receptor signaling is blocked within few hours of MV interaction (4), and we found that this is associated with a lack of its membrane recruitment in IL-2-stimulated Kit-225 cells (not shown). Similarly, membrane recruitment of this kinase and, most likely, other PH domain-containing proteins is impaired as well in T cells early after MV contact (Fig. 3). In support of this assumption, Vav failed to accumulate efficiently at the cell

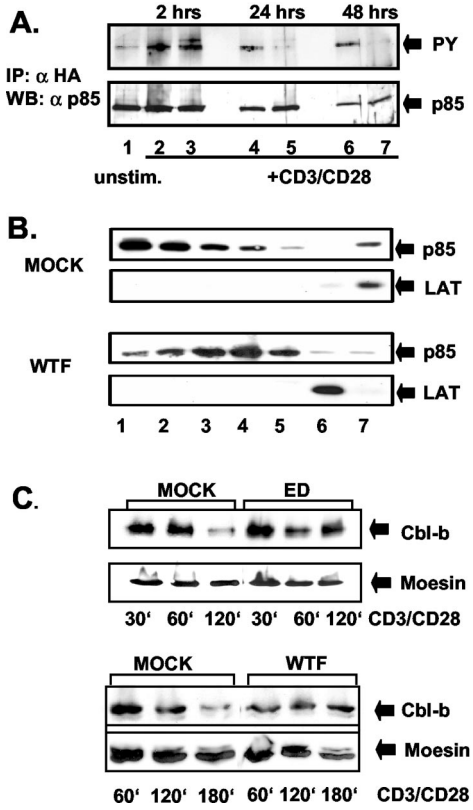

FIG. 5. Tyrosine phosphorylation and raft recruitment of PI3K regulatory subunit p85 and Cbl-b expression after MV contact. (A) pCG-p85αHA-nucleofected primary T cells were left unstimulated (lane 1) or mock (lanes 2, 4, and 6) or WTF (lanes 3, 5, and 7) treated, followed by CD3/CD28 stimulation for 30 min (lanes 2 to 7). Lysates were immunoprecipitated (IP) with an anti-HA-antibody, followed by antiphosphotyrosine immunoblotting (WB) (upper panel) and anti-p85 reblotting (lower panel). (B) Lysates from CD3/CD28-stimulated primary T cells pretreated with mock extract (top panel) or WTF (2 h at 4°C) (third panel) were fractionated and analyzed for p85 partitioning by immunoblotting. LAT protein was used as a raft marker (second and fourth panels). (C) Extracts were prepared from primary T cells mock treated or treated with ED or WTF (upper panels) for 2 h at 4°C prior to CD3/CD28 stimulation. Cbl-b expression was analyzed after the time intervals indicated by Western blotting (upper panels); a moesin-specific antibody served as a loading control (bottom panels).

membrane after TCR stimulation of these cells (Fig. 4C). We are currently investigating whether this might affect the activation of Rho GTPases. Using the GFP-tagged Akt-PH domain, we showed the lack of PIP_2 and PIP_3 generation in MV-treated T cells. Phosphoinositol-dependent proteins were found to be important in actin cytoskeleton reorganization, cell polarization, cell proliferation, and cell survival, particularly after TCR stimulation in mature T cells (9). Moreover, antigen-triggered T-cell proliferation is absolutely dependent on sustained PI3K activity and transport of PH domain-containing proteins to the immunological synapse (7, 14).

CD3/CD28-stimulation caused lipid raft recruitment of a major fraction of the Akt kinase in medium or mock-treated cells but not in T cells exposed to MV for 2 h (Fig. 4A and B). Membrane and lipid raft recruitment of this kinase and other PH domain-containing proteins is dependent on the PIP_2 and PIP_3 pool, which is regulated by the D-3 kinase activity of PI3K and lipid phosphatases such as PTEN and SHIP-1 (19). It is unlikely that MV interaction enhances the activity of these lipid phosphatases, since in Jurkat T cells, which lack these enzymes, inhibition of proliferation (29, 33) and Akt kinase activation (this study) were observed. Thus, it appeared more likely that MV already interferes with TCR-dependent activation of PI3K.

The PI3K regulatory subunit p85 acts also as an adaptor for targeting the holoenzyme to lipid rafts which are rich in the PI3K phosphoinositide substrates. After TCR engagement, CD28 is a target for p85 binding, and thus PI3K activity colocalizes with this protein (27). Our finding that p85 is normally phosphorylated early after MV treatment (Fig. 5A) yet fails to associate with lipid rafts upon stimulation (Fig. 5B) may be instrumental for understanding the observed inhibition of raft recruitment of PH domain-containing proteins such as the Akt kinase and thus of proliferative inhibition. In agreement with our findings (Fig. 2), inhibitors of PI3K such as wortmannin, which disrupt accumulation of PIP_3, did not immediately affect synapse formation or the accumulation of tyrosine-phosphorylated proteins at the synapse, similarly to MV (7, 14).

As p85 certainly does not require accumulation of PIP_3 in rafts, the mechanism of the lack of its recruitment has to be different. Importantly, we found that CD28-stimulated degradation of Cbl-b does not occur in MV-contacted T cells (Fig. 5C). In mature T cells, Cbl-b promotes ubiquitination of p85, modulates its recruitment to CD28 and TCR-CD3 complexes, and also regulates Akt phosphorylation levels (12). As also seen in our system, Cbl-b-targeted ubiquination did not affect early Ca mobilization and the accumulation levels of p85 (and also other Cbl-b binding proteins such as Lck and Vav) (7–9) (Fig. 2C and 5A) but rather affected protein translocation. It has been shown that Cbl-b negatively regulates the recruitment of p85 to CD28 and the TCR complex in transiently transfected T cells (11). Cbl-b is ubiquinated and thereby targeted for proteasomal degradation by CD28 costimulation (41), which does not occur in our system (Fig. 5C). Thus, MV obviously acts to prevent CD28-stimulated proteasomal degradation of Cbl-b. Together with direct lipid raft protein dynamic alterations, Cbl-b-mediated retention of p85 most likely provides the primary interference with TCR stimulation of MV interacting with lipid rafts and thereby recruitment of PH domain-containing proteins. Whether the subsequent, late effects on TCR-stimulated tyrosine phosphorylation and Ca mobilization occur downstream of or separately from these initial events will have to be resolved.

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