Interleukin-7 Compartmentalizes Its Receptor Signaling Complex to Initiate CD4 T Lymphocyte Response

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This article has been withdrawn by Thierry Rose, Blanche Tamarit, Pascal Lenormand, Abdelkader Namane, and Jacques Thèze. Anne-Hélène Pillet, Vincent Lavergne, and Jean-Claude Rousselle could not be reached. Fig. 4 was inappropriately presented. The withdrawing authors assert that the results of this article are valid.

Interleukin (IL)-7 is a central cytokine that controls homeostasis of the CD4 T lymphocyte pool. Here we show on human primary cells that IL-7 binds to preassembled receptors made up of proprietary chain IL-7Rα and the common chain γc shared with IL-2, -4, -9, -15, and -21 receptors. Upon IL-7 binding, both chains are driven in cholesterol- and sphingomyelin-rich rafts where associated signaling proteins Jak1, Jak3, STAT1, -3, and -5 are found to be phosphorylated. Meanwhile the IL-7-IL-7R complex interacts with the cytoskeleton that halts its diffusion as measured by single molecule fluorescence autocorrelated spectroscopy monitored by microimaging. Comparative immunoprecipitations of IL-7Rα heterodimers and IL-7-stimulated cells confirmed recruitment of proteins such as STATs, but many others were also identified by mass spectrometry. The IL-7Rα cytoplasmic domain is highly conserved among IL-7Rα receptors with IL-2, -4, -9, -15, and -21 receptors. Upon IL-7 binding, both chains are driven in cholesterol- and sphingomyelin-rich rafts where associated signaling proteins Jak1, Jak3, STAT1, -3, and -5 are found to be phosphorylated. Meanwhile the IL-7-IL-7R complex interacts with the cytoskeleton that halts its diffusion as measured by single molecule fluorescence autocorrelated spectroscopy monitored by microimaging. Comparative immunoprecipitations of IL-7Rα heterodimers and IL-7-stimulated cells confirmed recruitment of proteins such as STATs, but many others were also identified by mass spectrometry. The IL-7Rα cytoplasmic domain is highly conserved among IL-7Rα receptors with IL-2, -4, -9, -15, and -21 receptors. Upon IL-7 binding, both chains are driven in cholesterol- and sphingomyelin-rich rafts where associated signaling proteins Jak1, Jak3, STAT1, -3, and -5 are found to be phosphorylated. Meanwhile the IL-7-IL-7R complex interacts with the cytoskeleton that halts its diffusion as measured by single molecule fluorescence autocorrelated spectroscopy monitored by microimaging. Comparative immunoprecipitations of IL-7Rα heterodimers and IL-7-stimulated cells confirmed recruitment of proteins such as STATs, but many others were also identified by mass spectrometry.

IL-7 binds to its receptor, IL-7R, which is made up of two glycosylated chains anchored to the membrane by a single helical transmembrane domain (14): IL-7Rα and the common γ chain. IL-7Rα, also known as CD127 (65 kDa, 459 amino acids), is also shared by thymic stromal lymphopoietin (15, 16). The common γ chain (γc), also known as CD132 (56 kDa, 369 amino acids), is shared by IL-2, -4, -9, -15, and -21 (5). IL-7Rα is highly and γc weakly expressed at the surface of resting CD4 T cells. Their stimulation by IL-7 down-regulates the expression of IL-7Rα, which disappears from the cell surface after 12 h, and γc is rapidly shed from the cell surface (12) with a half-life of 1 h. γc is also expressed at high levels in thymus stromal cells where it regulates IL-7Rα expression (13). IL-7Rα acts as a high affinity binding site for IL-7, while γc acts as a low affinity binding site (14). The IL-7Rα cytoplasmic domain is highly conserved among IL-7Rα receptors with IL-2, -4, -9, -15, and -21 receptors. Upon IL-7 binding, both chains are driven in cholesterol- and sphingomyelin-rich rafts where associated signaling proteins Jak1, Jak3, STAT1, -3, and -5 are found to be phosphorylated. Meanwhile the IL-7-IL-7R complex interacts with the cytoskeleton that halts its diffusion as measured by single molecule fluorescence autocorrelated spectroscopy monitored by microimaging. Comparative immunoprecipitations of IL-7Rα heterodimers and IL-7-stimulated cells confirmed recruitment of proteins such as STATs, but many others were also identified by mass spectrometry. The IL-7Rα cytoplasmic domain is highly conserved among IL-7Rα receptors with IL-2, -4, -9, -15, and -21 receptors. Upon IL-7 binding, both chains are driven in cholesterol- and sphingomyelin-rich rafts where associated signaling proteins Jak1, Jak3, STAT1, -3, and -5 are found to be phosphorylated. Meanwhile the IL-7-IL-7R complex interacts with the cytoskeleton that halts its diffusion as measured by single molecule fluorescence autocorrelated spectroscopy monitored by microimaging. Comparative immunoprecipitations of IL-7Rα heterodimers and IL-7-stimulated cells confirmed recruitment of proteins such as STATs, but many others were also identified by mass spectrometry.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Table S1.

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The abbreviations used are: IL, interleukin; ACF, autocorrelated function; CCF, cross-correlated function; DRM, detergent-resistant membrane domain; FCS, fluorescence autocorrelated spectroscopy; FACS, fluorescence-activated cell sorting; IP, immunoprecipitation; Jak, Janus kinase; mAb, monoclonal antibody; pAb, polyclonal antibody; MS, mass spectrometry; SA488/633, streptavidin-Alexa Fluor 488/633; STAT, signal transducer and activator of transcription; TCR, T cell receptor; ERK, extracellular signal-regulated kinase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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involved in IL-7R compartmentalization at the level of a single complex in a single living primary human cell. This approach, at the molecular level and in real time, describes the very first steps in IL-7 response initiation that is crucial to CD4 T cell activation.

**MATERIALS AND METHODS**

**Human CD4 T Lymphocyte Purification**—Venous blood was obtained from healthy volunteers through the EFS (Etablissement Français du Sang, Centre Cabanels, Paris). Peripheral blood mononuclear cells were purified by density gradient centrifugation on Lymphoprep solution (Axis-Shield). CD3⁺/CD4⁺ NT cells were prepared from human peripheral blood mononuclear cells by separation on magnetic beads (CD4⁺ negative purification kit, Miltenyi Biotec). The enriched CD4 T cell population contained >95% CD3⁺/CD4⁺ cells. The recovered CD4 T cells were not activated as controlled by the absence of CD69 and CD25 expression.

Cell purity and preparations of IL-7R chain expression at the cell surface were analyzed by flow cytometry with labeled antibodies. Cells were harvested and resuspended in 50 μl of cytometer buffer (phosphate-buffered saline with 0.02% sodium azide and 5% fetal bovine serum) and labeled for 1 h at 4 °C with antibodies to CD4 (eBioscience). CD4 receptor expression was measured by flow cytometry in CD4 T cells using Cyan LXT™ cytometer (DakoCytomation). Data were analyzed with Summit version 4.1 software (Dako) and FlowJo version 8.3.3 software (Tree Star).

For cytokine activation, CD4 T cells were resuspended at 10⁶ cells/ml in RPMI 1640 medium (Gibco) supplemented with 5% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 5% fetal bovine serum) and labeled for 1 h at 4 °C with antibodies to CD4 (eBioscience). CD4 receptor expression was measured by flow cytometry in CD4 T cells using Cyan LXT™ cytometer (DakoCytomation). Data were analyzed with Summit version 4.1 software (Dako) and FlowJo version 8.3.3 software (Tree Star).

**FCS and FCCS Analysis of IL-7R Chain Assembly and Diffusion at the Surface of Living Cells**

**FCS and FCCS** measurements were made on living cells using an inverted laser scanning confocal microscope (LSM510), combined with a ConfoCor2 FCS system (Zeiss). Depth of field was spatially filtered through a 30–300-μm pinhole and fluorescence light was split into two detection channels with the following excitation/emission wavelengths: Alexa Fluor 488 (Invitrogen) (Ar, 488 nm/550–600 nm) and Alexa Fluor 633 (Invitrogen) (He/Ne, 633 nm/BP680 nm). FCS and image data were acquired and then analyzed by LSM software (Zeiss). The observation volumes of the LSM and the ConfoCor systems were aligned by bleaching spots with the ConfoCor in a dried layer of rhodamine-6G (Sigma) on a 0.16-mm glass slide from cross-hair positions in LSM images. Pinhole setting, volume overlap, and ConfoCor/LSM alignment were processed before each measurement session when laser power and temperature were at equilibrium. Pinhole x-, y-, and z-positions were carefully aligned for both channels 488 and 633 nm, and their confocal emission volume overlap was tuned by adjusting the collimator. Confocal volume was calibrated with rhodamine-6G and tested with beads as described under supplemental Fig. S1. Autocorrelation and cross-correlation functions were extracted from fluorescence intensity fluctuations during 30–60-s acquisition times and fitted to three-, two-, or three-/two-dimensional mixed mathematical models according to Ref. 24 with Origin software (OriginLab).

Three-dimensional diffusions of rhodamine-6G, streptavidin-Alexa 488 (SA488, Invitrogen), streptavidin-Alexa 633 (SA633, Invitrogen), IgG-biotin-streptavidin-Alexa 488 (mAb-SA488), and IL-7-biotin-streptavidin-Alexa 633 (IL-7b-SA633) were analyzed in RPMI medium at 37 °C. Autocorrelation functions (ACF) of fluorescent particle three-dimensional diffusion were fitted with the following model,

\[
G(\tau) = 1/N \left[ 1 - f_{\text{triplet}} + f_{\tau/(\tau/\gamma_{\text{triplet}})} \right] \left[ 1 - f_{\text{triplet}} \right] \sum \left[ f(1 + \left( \frac{\tau}{\tau_0} \right)^{2/3}) \right]
\]

where \( N \) is the number of fluorescent molecules in the actual detection volume defined as \( V = \pi r^2 h \), and \( s \) is the structural parameter representing the shape, as derived from the ratio between its axial and lateral radii \( s = \frac{h}{r} \). \( f_{\tau_0} \) is the component number, \( f_{\tau_1} \) and \( f_{\tau_2} \) the fractional process and diffusion time of particle in two component state, \( f_0 \) and \( f_1 \) the fractional process and diffusion time of fluorescent component (24). Below 50 × 10³ and beyond 150 × 10³ 2 values.

In addition, the volume intensity fluctuations during 30–60-s acquisition times and fit-cross correlation functions were extracted from fluorescence light was split into two detection channels with the following excitation/emission wavelengths: Alexa Fluor 488 (Invitrogen) or biotinylated anti-IL-7R (rAb/IL-7) tagged with streptavidin-Alexa 633 (SA488, Invitrogen), streptavidin-Alexa 633 (SA633, Invitrogen), IgG-biotin-streptavidin-Alexa 488 (mAb-SA488), and IL-7-biotin-streptavidin-Alexa 633 (IL-7b-SA633) were analyzed in RPMI medium at 37 °C. Autocorrelation functions (ACF) of fluorescent particle three-dimensional diffusion were fitted with the following model,
streptavidin-Alexa 633 (IL-7b-SA633) in a 1:4 molar ratio as well to avoid cytokine aggregation. Receptor diffusion measurements were acquired within a 10–30 min time frame in culture medium to minimize internalization effects and IgG-induced receptor aggregation. Confocal volumes in the FCS optical system and imaging laser scanning microscope were adjusted and centered on the cytoplasmic membrane. All images and FCS data were acquired at 37°C using a thermostated dish holder and objective ring. Ten 30-s acquisitions were recorded at 6 pin-hole values over 10 points spread over a 3×3-μm square of a single cell immobilized on poly-l-lysine-coated glass slides at the bottom of the dish. Briefly, autocorrelation curves, $G(\tau)$, were fitted with one up to three components: one fast three-dimension diffusion based component (free mAb-SA or IL-7b-SA) characterized by its diffusion time in the confocal volume ($\tau_{0,fast}$) and described by Equation 1, then one intermediate ($\tau_{D,inter}$) and one slow ($\tau_{D,slow}$) two-dimensional diffusion-based component as described by Equation 3, corresponding to the membrane-embedded receptor chains in different aggregation states.

$$G(\tau) = 1/N [1 - f_{f_{triplet}} + f_{f_{triplet}} e^{-\tau_{0}/\tau_{D,fast}}] / [1 - f_{f_{triplet}} \sum f_j [1 + (\tau/\tau_j)^{-1/2}]]$$ (Eq. 3)

Overall the model used with 3 components is given by Equation 4.

$$G(\tau) = 1/N [1 - f_{f_{triplet}} + f_{f_{triplet}} e^{-\tau_{0}/\tau_{D,fast}}] / [1 - f_{f_{triplet}} \sum f_j [1 + (\tau/\tau_j)^{-1/2}]] + f_{f_{inter}} [1 + (\tau/\tau_{D,inter})^{-1/2}] + f_{f_{slow}} [1 + (\tau/\tau_{D,slow})^{-1/2}]$$ (Eq. 4)

where $f_{f_{triplet}}$, $f_{f_{inter}}$, and $f_{f_{slow}}$ are relative fractions of component populations. Effective diffusion rates were calculated from the linear regression of $\pi/\omega_0^2$ and the intercept at the waist origin, $\omega_0^2 = 0$ in Equation 3, multiplied by the diffusing particles in $V_{eff}$ from the extracellular origin at the origin of autocorrelation functions.

$$G(\tau = 0) = 1/N$$ (Eq. 5)

Autocorrelation functions were normalized for comparison with Equation 6.

Normalized $G(\tau) = N(G(\tau) - 1)$ (Eq. 6)

Cross-correlation functions (CCF) between IL-7Rα-mAb-SA488 and IL-7b-SA633 were fitted using the following three-dimensional (Equation 7) and two-dimensional (Equation 8) diffusion models, respectively.

$$G_{ij}(\tau) = 1/N [1 - f_{f_{triplet,i}} + f_{f_{triplet,i}} e^{-\tau_{0,i}/\tau_{D,fast,i}}] / [1 - f_{f_{triplet,i}}] [1 - f_{f_{triplet,j}}] f_{f_{triplet,i}} [1 + (\tau/\tau_i)^{-1/2}] + f_{f_{triplet,j}} [1 + (\tau/\tau_j)^{-1/2}]$$ (Eq. 7)

$$G_{ij}(\tau) = 1/N [1 - f_{f_{triplet,i}} + f_{f_{triplet,i}} e^{-\tau_{0,i}/\tau_{D,fast,i}}] / [1 - f_{f_{triplet,j}}] [1 - f_{f_{triplet,i}}] [1 - f_{f_{triplet,j}}] f_{f_{triplet,i}} [1 + (\tau/\tau_i)^{-1/2}] + f_{f_{triplet,j}} [1 + (\tau/\tau_j)^{-1/2}]$$ (Eq. 8)

where $\tau_{ij}$ is the diffusion time of the complex IL-7Rα-mAb-SA488-IL-7b-SA633 considered as a unique species carrying both fluorophores mostly anchored to the membrane. SA488/SA633 fluorescence cross-talk was corrected from a single labeling experiment of the same system recorded in both channels (24).

Among our major concerns was receptor aggregation induced by antibodies or streptavidin, and receptor internalization. Diffusion plots (Equation 2) give control of receptor chain aggregation slowing down their diffusion with increasing time: the plot is curving up when acquisition starts from small to large $\omega_0^2$ as observed for diffusion of IL-7Rα labeled with the primary-secondary antibody complex mAb-sAb-A488 beyond 10 min of acquisition time and beyond 20–30 min with mAb-SA488. Controls were done with either free unlabeled streptavidin, biotin (Sigma), or mouse IgG added in culture medium. Usual conditions applied to lower receptor internalization were unfavorable: decrease of temperature affected membrane fluidity and cytoskeleton adhesion, and deoxyglucose inhibited the undifferentiated receptors affected receptor compartmentalization. Descriptions of the diffusion plot (Equation 2) for mem- branes affected by internalization were published in a Sucrose Gradient—Cell Lysate Ultracentrifugation through a Sucrose Gradient—Cell Signaling

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the complex by analyzing the cross-correlation between IL-7Rα·mAb-SA488 diffusion and the diffusion of biotinylated IL-7 labeled with SA633 (IL-7b·SA633) (Fig. 1E, curve h, and G): $D_{\text{eff}} = 0.024 \pm 0.0038 \, \mu m^2/s$, 7.2-fold slower than $\alpha$-yG and 9-fold than $\alpha$ (Fig. 2B). As can be seen in Fig. 2, A and B, extrapolation of the IL-7Rα-yG linear regression crosses the y axis below the origin before ($\tau_0 = -62 \pm 9.8 \, ms$) and after IL-7 binding ($\tau_0 = -242 \pm 38 \, ms$). $\tau_0$ was described by Marguet and colleagues (25) as the confinement time assessing the retention duration of particles by the cytoskeleton meshwork fencing reduced area below the plasma membrane. $\tau_0$ increases 4-fold after IL-7-binding, suggesting not only collision of the receptor complex but also tighter interactions with cytoskeleton components.

Mechanism Involved in IL-7R Compartmentalization—IL-7 binding slowed down the diffusion rate of the receptor and increased its confinement time. In attempts to demonstrate that the cytoskeleton is involved in confinement time and the diffusion brake, we used a drug that depolymerizes actin fibers: CytD. Fig. 3A shows that CytD did not significantly affect the diffusion rates of IL-7-free receptors: $D_{\text{eff}} = 0.227 \pm 0.030 \, \mu m^2/s$, $\tau_0 = +2.4 \pm 0.4 \, ms$ for 10 mm CytD compared with $D_{\text{eff}} = 0.173 \, \mu m^2/s$, $\tau_0 = -62 \, ms$ without CytD. By contrast, a CytD dose-dependent increase of IL-7R diffusion was noted in the presence of IL-7: $D_{\text{eff}} = 0.210 \pm 0.047 \, \mu m^2/s$, $\tau_0 = +8.1 \pm 1.3 \, ms$ for 0.024 mm CytD compared with $D_{\text{eff}} = 0.173 \, \mu m^2/s$, $\tau_0 = -242 \, ms$ without CytD. This increase suggests that the receptor complex is no longer constrained with the cytoskeleton and is released from its confinement state. Sphingomyelinase and cholesterol oxidase, which inhibit lipid raft formation, had no significant effect on receptor slowing in the presence of its ligand (Fig. 3B): $D_{\text{eff}} = 0.026 \pm 0.0036 \, \mu m^2/s$, $\tau_0 = -182 \pm 26 \, ms$. However, sphingomyelinase and cholesterol oxidase did have a noticeable effect after CytD treatment as the IL-7-IL-7R complex was once more freely diffused, as observed for the ligand-free receptor: $D_{\text{eff}} = 0.349 \pm 0.047 \, \mu m^2/s$, $\tau_0 = +11.4 \pm 1.7 \, ms$ (Fig. 3C).

IL-7-IL-7R Is Extracted into Detergent-resistant Microdomains when Activated—As sphingomyelinase and cholesterol oxidase affect the IL-7-IL-7R diffusion rate in the presence of CytD, this suggests that as well as interacting with the cytoskeleton, the receptor is also interacting with lipid rafts or proteins embedded in these rafts. To investigate receptor chain partition inside and outside lipid rafts upon ligand binding, we extracted DRM by ultracentrifugation in sucrose gradients. We lysed the CD4 T cells with 0.5% Triton X-100, removed by centrifugation unlysed cells and organelles, and separated the insoluble and soluble membrane fractions by ultracentrifugation on a sucrose viscosity gradient according to their sedimentation velocities. By using flotilltin-1 as a fraction marker of DRM microdomains, we demonstrated, in the Western blot shown in Fig. 4, that IL-7Rα and the yG chain are located in Triton-solubilized fractions 13–17 before IL-7 bind-
Preserved in Triton cell-lysis buffer. We therefore investigated IL-7R before and after IL-7 binding, and their interactions are—It was clear that some proteins are carried by IL-7 Binding

Effect of the cytoskeleton and lipid rafts on IL-7R diffusion. A, IL-7−free IL-7Rα, γc, and IL-7Rαγc diffusion by FCS/FCCS. The following diffusion times, \( t_0 \) (in 10^3 s), in the presence of IL-7−biotin-streptavidin-A633 are plotted versus the surface area \( \omega_0^2 \) intercepted by the confocal volume (in 10^3 nm^2). ACF of IL-7RαmAbb-SA488 in the absence of IL-7− (C), CCF of IL-7RαmAbb-SA488 with γc-anti-γc-mAbb (\( \gammac ACF \)), and 10 \( \mu M \) CytoD (\( \bullet \)). Slopes of the linear regression give effective diffusion rates, \( D_{eff} \), and intercepts at the y axis extrapolate \( t_0 \). Error bars give S.E.

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To characterize the role played by receptor partition in sig-

ing, and in insoluble fractions 6–10 after IL-7 binding. This appearance of IL-7R chains in DRM domains suggests that the receptors are driven into lipid rafts upon IL-7 binding, or that lipid rafts are formed around the cytokine-bound receptor.

To characterize the role played by receptor partition in sig-

naling, we checked whether Jak/STAT phosphorylation was associated with receptors inside or outside DRM. To do this, we immunoprecipitated proteins with anti-IL-7Rα: 1) from pooled fractions 6–10 (DRM), or 2) pooled fractions 13–17 (soluble fraction of the membrane) of the IL-7−stimulated cell lysate samples. Protein phosphorylation on the Western blots was detected using anti-Tyr(P). Fig. 5 shows that proteins with IL-7−induced phosphorylation of Tyr were mainly located inside DRM. More specifically, phosphorylated Jak1, Jak3, STAT3, and STAT5 after IL-7 activation were found in DRM, with barely traces outside.

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the protein cortege pulled down with the receptor by immuno-precipitation using anti-IL-7R/H9251 from lysed CD4 T lymphocytes. The first step consisted in checking the presence of proteins involved in IL-7R signaling that were separated from the IP complex by heat denaturation in SDS and migration on one-dimensional SDS-PAGE. Protein-specific Western blots are shown in Fig. 6. IL-7R/H9251 was detected as a control. γc was found in cells activated by IL-7 and to a lesser extent in resting cells. This demonstrated that γc interacts spontaneously with IL-7R/H9251 at the surface of resting CD4 T lymphocytes prior to becoming embedded in lipid rafts. IL-7 stabilizes the IL-7R-γc interaction as suggested by the darker band of pulled down γc.

The same amount of Jak1 was carried out before and after IL-7 binding, and this was consistent with the constitutive binding of Jak1 onto the IL-7R/H9251 cytoplasmic domain. The Jak3 band was darker in the activated signaling complexes and its quantity correlated to the amount of γc. Jak1 and Jak3 are resident proteins on IL-7R chains and some complexes were fully preassociated prior to IL-7 binding. As expected, STAT1, STAT3, and STAT5 were pulled down only after IL-7 binding as STAT recruitment after IL-7-induced phosphorylation of the IL-7R/H9251 cytoplasmic domain on Tyr456 provides a STAT binding site.

Proteins ERK1 and ERK2 were also clearly recruited on the signaling complex after IL-7 binding to IL-7R. As IL-7R interaction with cytoskeleton was expected, recruitment of actin (microfilament), α-tubulin (microtubule), ezrin and moesin (FERM linking rafted proteins with cytoskeleton) were tested and the presence of all four proteins was confirmed after IL-7 activation (Fig. 6).

### FIGURE 4

**IL-7Rα and γc are found in DRM after IL-7 stimulation of CD4 T cells.** CD4 T lymphocyte lysates were loaded on a 5–40% sucrose gradient and divided into 18 fractions after 16 h of centrifugation at 50 krpm at 4 °C. Fractions: 1 left, tube top = 5%; 18 right, tube bottom = 40%) were loaded on SDS-PAGE (7% acrylamide-bisacrylamide). Flotillin, IL-7Rα, and γc were located in the membrane fractions by immunoblotting. Fractions corresponding to DRM are indicated above the membrane strip according to flotillin distribution.

### FIGURE 5

**Phosphorylated Jaks and STAT5 are found mainly in DRM after IL-7 stimulation of CD4 T cells.** Materials were prepared as described in the legend to Fig. 4. a, after centrifugation, fractions 6 to 10 were pooled to provide a “DRM” sample and fractions 13 to 17 were pooled to form a “solubilized” sample. Both samples were loaded on SDS-PAGE (7% acrylamide-bisacrylamide). Tyr-phosphorylated proteins Tyr(P) (b and c), pJak (d and e), pSTAT3 (f and g), and pSTAT5 (h and i) were revealed by immunoblotting.

### FIGURE 6

**Proteins immunoprecipitated with IL-7Rα before and after IL-7 stimulation of CD4 T cells.** Proteins were immunoprecipitated with anti-IL-7Rα from CD4 T lymphocyte lysate and separated on SDS-PAGE (7% acrylamide-bisacrylamide). Corresponding bands were cut out of images of specific immunoblots from non-stimulated (NS) and IL-7-stimulated (+IL-7) cells. A, “IL-7-recruited” protein...
PROTEINS. Proteins were digested in trypsin, eluted, and analyzed by combined MS and MS/MS from MALDI-TOF/TOF procedures. The 109 proteins identified from at least 5 sequenced peptides specific to the protein sequence in the Uniprot data base are detailed in supplemental Table S1. They were then sorted according to the increase in spot staining intensity after IL-7 binding. 78 proteins were increased by a factor of 4 or more, whereas 26 proteins were increased by a factor from 2 to 3.9. We also noted that four proteins were decreased after IL-7 binding.

Among the 109 proteins identified, Table 1 lists the 78 proteins recruited by the IL-7R signaling complex after IL-7 binding, i.e. that were at least 4-fold more abundant in the immunoprecipitated complex after IL-7 binding than before. Two-thirds of the pulled down proteins (48/78) are involved in the cytoskeleton (42/78) or have been described as associated with lipid rafts (6/78). None of the proteins in the IL-7R signalingosome and none of those found by Western blotting (Fig. 6) in the same IP complex preparation were among the proteins identified until the cut off was lowered to select peptides with m/z peaks bellow the top 15 most intense. The concentration of abundant proteins overwhelmed those present at low levels. This group included actin and its filament assembly (gelsolin, cofillin-1, -2, and -3), profilin, actin capping proteins, and coactosin) and membrane anchors or intermediate linkers (vinculin, zyxin, ezrin, moesin), proteins involved in microfilament polymerization (HSP70), proteins involved in calcium-induced recruitment by the cytoskeleton (calmodulin, calreticulin, sorcin), and actin-binding linkers and carriers (myosin, tropomyosin, plastin-2, and derbrin-like). Microtubule subunits (\(\alpha\) and 2-\(\beta\)-tubulin) were abundantly recruited upon IL-7 binding. We also found proteins known for their embedding in lipid rafts (integrin) and their interactions with the cytoskeleton through vinculin, moesin, plektasin, which interacts with phosphatidylinositol 4,5-bisphosphate in lipid rafts was also found. Certain recruited proteins are mainly involved in folding/unfolding and degradation processes (HSP, proteasome activator, thioredoxine, glutathione transferase, and disulfide isomerase) and oxidative-reduction reactions (peroxiredoxin and superoxide dismutase) and might be carried with the cytoskeleton.

Fig. 8 summarizes the IL-7-driven compartmentalization steps according to our IP analysis before (Fig. 7A) and after (Fig. 7B) IL-7 binding. Cytokine-free IL-7R diffuses freely with a reduced carriage (Jaks) at the surface of CD4 T lymphocytes, then upon cytokine binding IL-7R is recruited by the cytoskeleton and interacts with other raft-embedded proteins.

**DISCUSSION**

IL-7 induces a variety of responses *ex vivo* in CD4 T lymphocytes, e.g. cell activation, survival, and proliferation, mainly through \(\gamma\)c-signaling Jak/STAT, AKT/phosphatidylinositol 3-kinase, and mitogen-activated protein kinase pathways. Sig-
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**FIGURE 8. Sketch views of the IL-7R-signaling complex assembly upon IL-7 binding.** Left (NS conditions), the IL-7-free heterodimer IL-7Rα-γc is embedded in the lipid bilayer out of rafts; Jak1 and Jak3 are constitutively bound to their cognate cytoplasmic receptor chains. Right (IL-7 conditions), the complex IL-7-IL-7Rα-γc is embedded in the lipid raft. STAT is bound to IL-7Rα-γc-Jak1-Jak3. FERM proteins (E) connect IL-7Rα to F-actin. Integrin chains are linked to F-actin through praxillin (P) or talin (T) complexed to vinculin (V) and Arp2-3 (42). ABP represents actin-binding proteins, S symbolizes proteins inhibiting F-actin elongation, and T represents proteins involved in filament ramification. Tubulin assemblies and proteins unrelated to cytoskeleton are not represented.

Naling proteins involved have been mainly identified through their cytokine-induced phosphorylation and have been functionally grouped together in the IL-7R “signalosome” (4, 5, 23). However, the early IL-7 response has yet to be described at the molecular level of its receptor and interacting proteins. A large physical entity and are considered the “signalosome.” The mechanism involved in assembling the signalosome itself is unknown and the protein involved is unknown concerning the number of proteins involved. New time-resolved microimaging methods have now enabled us to analyze receptor assemblies on living primary cells revealing the story board of the early receptor response for molecules involved. New time-resolved microimaging methods have now enabled us to analyze receptor assemblies on living primary cells revealing the story board of the early receptor response for molecules involved. New time-resolved microimaging methods have now enabled us to analyze receptor assemblies on living primary cells revealing the story board of the early receptor response for molecules involved. New time-resolved microimaging methods have now enabled us to analyze receptor assemblies on living primary cells revealing the story board of the early receptor response for molecules involved.

In our work, we noted that two-thirds of the proteins recruited upon IL-7 binding have already been described as part of the cytoskeleton or are associated with lipid rafts. Cytoskeleton-associated proteins identified included microfilament and microtubule compounds, proteins regulating their polymerization and depolymerization, and intermediates such as myosin and tropomyosin. We also found proteins involved in cytoskeleton connection to the membrane (ezrin, moesin, and vinculin). These FERM proteins (for 4.1 protein, ezrin, radixin, and moesin) regulate the anchorage of plasma membrane proteins to actin cytoskeleton. These proteins have already been described in the regulation of signal transduction pathways (33, receptor almost halted its lateral diffusion and, in accordance with the criteria established by Marguet and colleagues (25, 29), receptor retention time in the cytoskeleton meshwork was increased at least 4-fold after IL-7 binding, suggesting not only collision of the receptor complex but also tighter interactions with cytoskeleton components in living cell cultures at 37 °C. This was first demonstrated by showing that the interaction was lost when IL-7-activated cells were treated with a cytoskeleton polymerization inhibitor (cytochalasin D). Our subsequent analysis by two-dimensional PAGE further supported these results as discussed below. IL-7 binding to its receptor also altered its distribution in detergent-resistant membrane nanodomains. This observation suggests that migration was taking place into lipid rafts or that lipid rafts were being formed around the receptor. We have therefore for the first time shown that phosphorylated Jak and STAT5 are associated with raft-embedded receptors. Interestingly, although lipid raft formation was not dependent on the cytoskeleton meshwork, receptor dissociation from lipid rafts was dependent on depolymerization of actin filaments.

Our studies of IP complex composition showed that three categories of proteins were pulled down with anti-IL-7Rα. First, we found proteins themselves, IL-7Rα and γc, as previously suggested (21, 23) and demonstrated for homolog cytokine and then STAT5 and ERK, some proteins were pulled down transiently in the presence of IL-7 binding. Second, some proteins were pulled down upon cytokine binding and were then released, for instance, STAT and ERK. The last category of proteins was pulled down only after IL-7 binding. Among proteins involved in IL-7 signaling pathways that were not found in the MS protein list according to the stringent selection criteria used for protein identification: at least 5 of the 15 most intense m/z peaks yielded a positive sequence match with the MS/MS analysis. However, the presence of several proteins was validated by Western blotting and many were found from MS lists using less stringent criteria. To date, among cytokine receptors only, the signaling complex associated with mouse IL-1R has been studied by proteomics (30). Interferon, prolactin, growth hormone, and erythropoietin are among those best resolved for their assembly at the molecular level, and although their signaling complexes have not yet been elucidated, pioneer works on epidermal growth factor receptor and prolactin receptor have suggested that machinery carried by the cytoskeleton might be involved (31, 32).

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34. Ezrin and moesin are well expressed in T cells, whereas radixin is low. FERM proteins interact with positively charged amino acid clusters in the juxtamembrane region of proteins embedded in lipid rafts. There is a putative FERM-binding domain at the juxtamembrane sequence of the cytoplasmic domain of IL-7Rα, characterized by several basic residues \( ^{265}KKRIPVKVSPDLHKKTLEHLCKKPRK \) in the extension of the helical transmembrane domain (240–264) (35) as well as found in IL-2Rβ \( ^{266}NCRNTGPWLLKVLKNTP-DPSK \) but not in γc. This FERM-binding domain could be involved in receptor recruitment of FERM proteins, anchoring the complex to the cytoskeleton. Full-length FERM proteins show a low level of binding activity to both membrane and actin. These inactive states are believed to be expressed by a masking mechanism in which the FERM domain binds the C-terminal half to suppress the actin filament and membrane binding activities (36–39). Biochemical studies have shown that phosphatidylinositol 4,5-bisphosphate also binds FERM domains and stimulates the binding of FERM proteins to their targets (39, 40). Interestingly, the FERM domains bind the Rho-specific GDP-dissociation inhibitor (RhoGDI) found among IL-7R-recruited proteins and accelerate the release of Rho to activate Rho-dependent processes (39, 41), suggesting involvement by the Rho signaling pathway.

Integrin αIIb receptor chain is among the most IL-7R-recruited proteins upon IL-7 binding. This receptor chain interacts with the extracellular matrix (fibronectinogen γ), has been described as embedded in lipid rafts, vinculin, which is also among the highly abundant proteins implicated in the IL-7R signaling cascade, but also a wide array of proteins associated directly or indirectly with the plasma membrane and intracellular membranes, and implicated in a variety of cell functions. Although the biological significance of the association between lipid rafts, the cytoskeleton, and the various proteins identified requires clarification, this study provides insight into the profound and far reaching changes induced in a cell by the triggering of surface receptors.

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