Insights into the Association of FcγRII and TCR with Detergent-Resistant Membrane Domains: Isolation of the Domains in Detergent-Free Density Gradients Facilitates Membrane Fragment Reconstitution[†]

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ABSTRACT: Plasma membrane rafts are routinely isolated as detergent-resistant membranes (DRMs) floating in detergent-free density gradients. Here we show that both the presence and exclusion of TX-100 during the density gradient fractionation have profound effects on the location of FcγRII and TCR in DRM fractions. The presence of TX-100 during fractionation promoted solubilization of non-cross-linked FcγRII when the receptor was insufficiently dissolved upon cell lysis. In the detergent-supplemented gradients, TX-100 micelles floated, further enhancing dissociation of FcγRII and TCR from DRMs and promoting a shift of the receptors toward higher-density fractions. Hence, fractionation of cell lysates over the detergent-containing gradients enables isolation of DRMs devoid of weakly associated proteins, like nonactivated FcγRII and TCR. On the other hand, in a detergent-free gradient, non-cross-linked FcγRII, fully soluble in 0.2% TX-100, was recovered in DRM fractions. Moreover, employment of the TX-100-free gradient for refractionation of intermediate-density fractions, derived from detergent-supplemented gradients and containing FcγRII and TCR, resulted in flotation of the receptors to buoyant fractions. An analysis of the TX-100 concentration revealed that after fractionation of 0.2% TX-100 cell lysates in the absence of detergent, the level of TX-100 in DRM fractions was reduced to 0.01%, below the critical micelle concentration. Therefore, fractionation of detergent cell lysates over detergent-free gradients can mimic conditions for a membrane reconstitution, evoking association of a distinct subset of membrane proteins, including FcγRII and TCR, with DRMs.

Sphingolipids and cholesterol in the plasma membrane are thought to undergo a phase separation from the otherwise glycerophospholipid-rich environment, and in doing so, they form lipid rafts (*1*, *2*). Proteins with saturated lipid moieties, such as glycosylphosphatidylinositol-anchored proteins and members of the Src family protein tyrosine kinases, acylated with myristate and palmitate, accumulate preferentially within these membrane domains (*3*−*7*). A palmitoylated integral membrane protein caveolin is located in raft-related structures named caveolae, the functions of which remain unclear (*8*). In cells of hematopoietic origin (lacking caveolae), the plasma membrane rafts are implicated in signal transduction through immunoreceptors. It is suggested that the receptors, including T cell receptor (TCR),[1] B cell receptor, Fcϵ receptor I (FcϵRI), FcαR, and FcγRII, associate with the rafts upon activation, where they are phosphorylated by the Src family kinases and trigger signaling cascades (*9*−*16*).

Attempts to isolate lipid rafts and the associated proteins have relied mainly on the insolubility of the rafts in cold, nonionic detergents, e.g., Triton X-100 (TX-100), and their flotation in density gradients yielding so-called detergent-resistant membranes (DRMs) (*17*). However, the results of these studies are inconsistent. The DRMs isolated from T lymphocytes were either rich in activated TCR (*10*, *11*) or devoid of the receptor (*18*, *19*). Moreover, while the same reports indicated that many downstream TCR signaling molecules such as Cbl, Syk, Vav, ZAP-70, and PLC-γ1 also accumulated in DRMs after receptor activation (*11*), other studies detected no Cbl and ZAP-70 localized to the DRMs (*19*). Furthermore, various amounts of activated FcϵRI were found in buoyant fractions of DRMs of RBL cells, which questioned the involvement of the rafts in the phosphorylation of the receptor (*20*). In an analogy to a problem of caveolae purification pointed out by Palade and colleagues (*21*), the discrepancies concerning the association of various proteins with rafts can result from differences in the detergent-based procedure applied for their isolation. In the majority of the studies, nonionic detergent cell lysates were fractionated over detergent-free density gradients (*9*−*11*, *14*, *17*, *19*). These conditions, however, can facilitate reconstitution of membranes, affecting the protein composition of isolated DRMs. In this study, we examine the effect of the exclusion of the detergent on the association of FcγRII and TCR with isolated DRMs.

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[1] Abbreviations: DRM, detergent-resistant membrane; GPI, glycosylphosphatidylinositol; FcαR, receptor IgA; FcγR, receptor IgG; FcϵRI, high-affinity IgE receptor; PC, phosphatidylcholine; TCR, T cell antigen receptor; TfR, transferrin receptor; TX-100, Triton X-100.

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MATERIALS AND METHODS

Isolation of DRMs by Gradient Ultracentrifugation. U937 monocytic cells and Jurkat T cells [both from American Type Culture Collection (ATCC)] were cultured, harvested, and treated with anti-FcγRII and anti-TCR antibodies in Hepes-buffered saline [125 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 10 mM glucose, 0.2% BSA, and 20 mM Hepes (pH 7.4)], essentially as described previously for U937 cells (*16*). Briefly, U937 cells (12.5×10^6 cells/mL) were exposed at 0 °C to anti-FcγRII mouse IgG2b, clone IV.3 (ATCC), purified from the hybridoma supernatant, and conjugated with biotin, while Jurkat cells were treated at 0 °C with biotin-conjugated anti-CD3ϵ mouse IgG1 (Ancell). After 30 min, the cells were either lysed or incubated with goat anti-mouse IgG (Sigma) (0 °C, 30 min) to induce cross-linking of FcγRII and TCR. In a set of experiments, U937 cells not exposed to the antibodies were used. Cells (12.5×10^6, unless indicated otherwise) were lysed for 10 min in 200 μL of ice-cold hypotonic buffer [2 mM EDTA, 2 mM EGTA, 30 mM Hepes (pH 7.4), 1 mM Na_3VO_4, 50 μM PAO, 1 mM PMSF, and a cocktail of protease inhibitors from Boehringer]. Subsequently, the lysates were supplemented with 150 mM NaCl and TX-100 at the concentrations given in the Results, and 10 min later, the samples were passed five times through a 25 gauge needle. After clarification (30 s at 480*g* and 4 °C), 200 μL of the lysates was mixed with 400 μL of 60% OptiPrep and complemented with 10% sucrose and 150 mM NaCl. The samples were overlaid with 400 μL of 30%/25%/20% OptiPrep containing 10% sucrose in buffer A. On the top, 300 μL of buffer A and 10% sucrose was loaded. Buffer A was composed of 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 30 mM Hepes (pH 7.4), and the protease and phosphatase inhibitors as in the lysis buffer, with or without TX-100. Gradients were centrifuged for 3.5 h at 4 °C and 170000*g* (RCM 100 ultracentrifuge, Sorvall). Seven fractions of 300 μL collected from the top of the gradient were weighed to estimate the density. In a series of experiments, the secondary gradient centrifugation of the fractions was conducted. For this purpose, 60 μL of the indicated fractions was combined and gently mixed under N_2 for 30 min at room temperature, adjusted to 40% OptiPrep and 10% sucrose, and fractionated over a detergent-free or TX-100-containing step gradient as described above.

To fractionate TX-100 cell lysates by sucrose gradient ultracentrifugation, 200 μL of the clarified lysates was mixed with 200 μL of 80% sucrose in 100 mM NaCl and 30 mM Hepes (pH 7.4). Sucrose step gradients were formed in centrifuge tubes (Sorvall) by adding 300 μL of 60% sucrose, 400 μL of 40% sucrose (containing the cell lysates), and 800 μL of 30%, 300 μL of 20%, and 300 μL of 5% sucrose, all in buffer A, with or without TX-100. The gradients were fractionated for 16 h at 4 °C and 170000*g* (RCM 100 ultracentrifuge) and divided into seven fractions of 300 μL collected from the top of the gradients.

Analysis of the Solubility of FcγRII in TX-100. U937 cells ($12.5-1.25 \times 10^6$) were either left untreated with antibodies (FcγRII nonprimed), exposed to anti-FcγRII mouse IgG-biotin alone (non-cross-linked FcγRII), or incubated additionally with goat anti-mouse IgG (cross-linked receptor). Cells were lysed in 200 μL of ice-cold lysis buffer supplemented with 0.2% TX-100, according to a procedure applied for isolation of DRMs by gradient ultracentrifugation. After clarification (30 s at 480*g* and 4 °C), lysates (200 μL) were subjected to high-speed centrifugation according to the method described in ref *22* (1 h at 250000*g* and 4 °C, Sorvall Discovery M120 centrifuge). Obtained supernatants were collected, and pellets were resuspended in 200 μL of buffer A without TX-100. Equal volumes of paired pellets and supernatants were subjected to SDS−PAGE. Where noted, 200 μL of supernatants was subjected to OptiPrep density gradient centrifugation, as described above.

Measurement of the TX-100 Concentration. To estimate the concentration of TX-100 in density gradient fractions, the method of Eibl and Lands (*23*) for inorganic phosphorus quantitation was adapted. In our modification, the turbidity produced by TX-100 in the presence of the phosphomolybdate complex was proportional to the detergent concentrations in the range of 0.0005−0.005%. The assay mixture contained 100 μL of 200 mM NaH_2PO_4 and a series of volumes of gradient fractions (5−100 μL) to match the TX-100 concentration within the linear range of measurements. The volume of the mixture was adjusted to 800 μL with H_2O. The reaction was started by adding 200 μL of 2.5% $(NH_4)_6Mo_7O_{24}$ in 6 N H_2SO_4, and 20 min later, the turbidity was measured at 660 nm in a Shimadzu UV-1201 spectrophotometer. Standard curves were prepared with known concentrations of TX-100 in buffer A, with or without OptiPrep and sucrose. Preliminary experiments showed that 5−40% OptiPrep and 5−60% sucrose did not interfere with the assay. This was in contrast to attempts to determine TX-100 content in fractions by measurement of their absorption at 275 nm due to the contribution of the high-UV absorption of OptiPrep.

TX-100 micelles containing phosphatidylcholine (PC) were prepared as follows. Appropriate amounts of PC in a chloroform/methanol mixture (3:1) were supplemented with 1 μL of [^{14}C]PC (0.025 μCi, Amersham), dried under nitrogen, then suspended in 200 μL of 0.2% TX-100 ($\approx$3.2 mM) in buffer A, and vortexed for 2 min. The final concentration of PC in the solutions was 0.32 or 0.64 mM. In control samples, no PC was added. The solutions were mixed with 400 μL of 60% OptiPrep and 10% sucrose and overlaid with density gradients containing 0.2% TX-100. Fractionation was conducted as described above. In collected fractions, the TX-100 concentration was estimated and radioactivity was measured in a Beckman scintillation counter. Basically, the same procedure was employed to prepare mixed micelles containing various amounts of PC with a constant TX-100 concentration. After dilution to 0.002% TX-100 (corresponding to 32 μM), these mixtures were used for examination of the influence of the presence of PC on TX-100 concentration measurements.

Immunoprecipitation. Gradient fractions (250 μL) or cell lysates (6.25×10^6 cells/200 μL of 0.05−0.2% TX-100 in buffer A) were supplemented with 2 μg of rabbit anti-Lyn IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated overnight at 4 °C. In control samples, the anti-Lyn antibody was omitted. Later on, goat anti-rabbit IgG Immunobead Reagent (Bio-Rad) was added (200 μL of a 2.5% solution per sample) for incubation for 3 h at room temperature The beads were preincubated with 5% nonfat milk and 3% BSA in Tris-buffered saline [120 mM NaCl and 20 mM Tris (pH 7.5) (TBS)] with 0.05% Tween-20 and

washed with buffer A supplemented either with 0.05 or 0.2% TX-100 or without the detergent, as indicated. The immunoprecipitates were washed seven times in buffer A with or without TX-100, resuspended in 28 μL of 2× SDS sample buffer, boiled, and subjected to SDS−PAGE.

Gel Staining and Immunoblotting. Immunoprecipitates and supernatant and pellet fractions from high-speed centrifugation and gradient fractions (20 μL) were loaded onto 10% reducing SDS−PAGE gels, and separated proteins were either stained with Silver Stain Plus reagent (Bio-Rad) according to the manufacturer's instructions or transferred to nitrocellulose and probed with rabbit anti-Lyn IgG, mouse anti-Lck IgG, clone Lck-01 (kindly provided by V. Horejsi, Institute of Molecular Genetics, Prague, Czech Republic), rabbit anti-transferrin receptor (TfR) labeled with biotin (Ancell), and the mouse anti-Na^+,K^+-ATPase α1 subunit (Sigma). Mouse anti-Lyn IgG (Santa Cruz Biotechnology) was used to reveal Lyn in immunoprecipitates. As secondary antibodies, goat anti-rabbit IgG, goat anti-mouse IgG (Boehringer), and goat anti-biotin IgG (Sigma), all conjugated with peroxidase, were applied. To detect biotin-labeled anti-FcγRII and anti-CD3ϵ antibodies, which reflected the distribution of FcγRII and TCR in the OptiPrep gradient fractions and immunoprecipitates, the goat anti-biotin peroxidase-conjugated IgG was used for the immunoblotting. When FcγRII nonprimed with antibody was examined, rabbit anti-FcγRII (kindly provided by J.-L. Teillaud of the Center de Recherches Biomedicales des Cordeliers, Paris, France) was applied for detection of the receptor. CD55 was detected by dot-blot analysis of 3 μL of gradient fractions with the use of rabbit anti-CD55 IgG (Santa Cruz Biotechnology) and anti-rabbit peroxidase-bound IgG. Immunoreactive bands were visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce) in a Fluor-S MultiImager and quantified densitometrically using Quantity One software (Bio-Rad). The data that are presented are representative of at least three independent experiments.

RESULTS

Non-Cross-Linked FcγRII Associates with the DRM Fraction in a TX-100-Dependent Manner. To analyze the influence of conditions of raft isolation on the association of FcγRII with DRMs, U937 cells pretreated with anti-FcγRII antibody were solubilized with TX-100 at concentrations ranging from 0.05 to 0.5% (w/v). Binding of the anti-FcγRII antibody alone neither induces noticeable clustering of the receptor nor triggers tyrosine phosphorylation of FcγRII and downstream proteins (*16*, *24*). The lysates were subjected to ultracentrifugation over OptiPrep density gradients either containing TX-100 at a concentration corresponding to that used for cell lysis or devoid of the detergent. In the latter case, up to 80% of non-cross-linked FcγRII floated up to low-density gradient fractions 1 and 2 (1.06−1.13 g/mL), except for lysis in 0.5% TX-100 which reduced the amount to 45.5 ± 5.1% ($n = 4$) (Figure 1A, left panel). In fractions 1 and 2 of the gradients, 70−83% of the Lyn kinase and ~95% of the GPI-linked protein CD55 also accumulated, suggesting that the lightest gradient region contained DRMs (Figure 1A, left panel). When gradient fractionation was carried out in the presence of 0.05% TX-100, FcγRII still accumulated in fractions 1 and 2 of the gradient; however, at 0.1% TX-100, only 43.3 ± 1.8% of the receptor ($n = 3$) was retained with DRMs, and at 0.2−0.5% TX-100 in the gradients, FcγRII was recovered only in high-density fractions 4−7 (1.19−1.27 g/mL) (Figure 1A, right panel). In contrast, most of the Lyn kinases and CD55 remained present in fractions 1 and 2, indicating that the integrity of DRMs was not significantly affected under these conditions (Figure 1A, right panel). TfR and Na^+,K^+-ATPase were recovered exclusively in high-density fractions 5−7, independent of the TX-100 concentration used for cell lysis and fractionation, as shown in Figure 1A for 0.2% detergent.

Fractions 1 and 5 of gradients obtained from 0.2% TX-100 cell lysates were next subjected to Lyn immunoprecipitation and analyzed for the presence of FcγRII. The receptor was immunoprecipitated together with Lyn complexes of buoyant fraction 1, however, only when the fractionation and immunoprecipitation procedures were performed without TX-100 (Figure 1B). The results suggest that the presence of TX-100 during the isolation of DRMs strongly affects the ability to detect their interaction with non-cross-linked FcγRII. To confirm this assumption, fractions 1, 2, 4, and 5 from the 0.2% TX-100 gradient were mixed and refractionated by density gradient ultracentrifugation without or with 0.2% TX-100 (Figure 1C). Input fractions 1 and 2 were rich in Lyn and devoid of FcγRII, while fractions 4 and 5 contained large amounts of the receptor and some Lyn (see Figure 1A). After the second fractionation in the presence of 0.2% TX-100, the distribution of FcγRII and Lyn closely resembled their original patterns, with the receptor accumulating in high-density fractions 4−7. However, when the second fractionation was conducted through a detergent-free gradient, 72.7 ± 4.6% of FcγRII ($n = 3$) floated up to fractions 1 and 2 of the gradient (Figure 1C), suggesting that under these conditions FcγRII from fractions 4 and 5 can associate with preexisting DRMs. Accordingly, the receptor was found in Lyn immunoprecipitates obtained from fractions 1 and 2 of the second gradient devoid of TX-100 (Figure 1D). On the other hand, the second centrifugation of fractions 4 and 5 alone over the detergent-free gradient also resulted in shifting of 47.7 ± 1.7% of FcγRII ($n = 3$) to buoyant fractions 1 and 2, despite the fact that the distribution of Lyn was confined to fractions 5−7 of this gradient. Incorporation of 0.2% TX-100 into the gradient precluded the receptor shifting to the buoyant fractions (Figure 1E). These results indicate that FcγRII, recovered in intermediate fractions 4 and 5 after primary gradient centrifugation of cell lysates in the presence of TX-100, can be located in mixed micelles with lipids, however, separated from Lyn. Depletion of TX-100 from intermediate fractions 4 and 5 during the subsequent fractionation over the detergent-free gradient resulted in FcγRII reconstitution into membrane fragments formed from lipids of fractions 4 and 5. Fully solubilized transmembrane proteins, including TfR, Na^+,K^+-ATPase, a small population of Lyn, and FcγRII, revealed in high-density fractions 6 and 7 of the first density gradient, did not float up during the secondary fractionation regardless of the presence of TX-100 (Figure 1E).

Solubility of FcγRII in TX-100. The results shown in panels C−E of Figure 1 indicate that fractionation of TX-100 cell lysates over detergent-free gradients can facilitate reconstitution of primarily solubilized FcγRII into membrane fragments, recovered eventually in low-density fractions 1 and 2. Alternatively, one might expect that FcγRII was

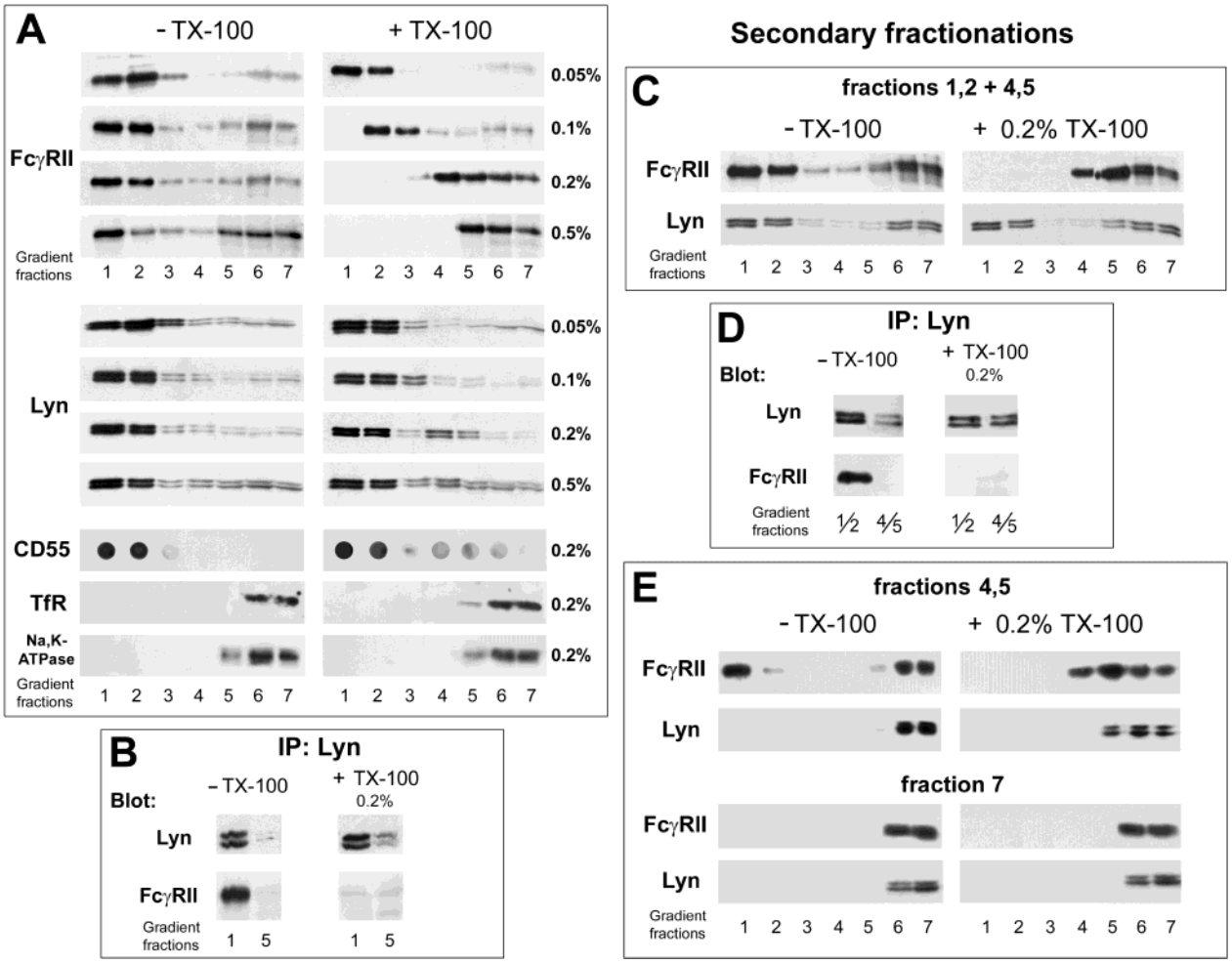

FIGURE 1: Effect of TX-100 on the association of non-cross-linked FcγRII with DRMs. U937 cells (12.5×10^6) were pretreated with biotin-labeled anti-FcγRII antibody (0 °C for 30 min) and then lysed in 200 μL of buffer containing 0.05−0.5% TX-100. The lysates were subjected to ultracentrifugation over OptiPrep density gradients (from 0 to 40%) either devoid of the detergent or containing amounts of TX-100 corresponding to that in the lysis buffer. (A) Distribution of non-cross-linked FcγRII, Lyn, CD55, TfR, and Na^+,K^+-ATPase in gradient fractions obtained after fractionation of TX-100 cell lysates in detergent-free (left panel, −TX-100) and TX-100-containing gradients (right panel, +TX-100). Concentrations of TX-100 used for cell lysis and included in the gradient, when indicated, are shown at the right. (B) Immunoprecipitation of Lyn from fractions 1 and 5 of OptiPrep density gradients obtained either without (left panel) or with addition of 0.2% TX-100 to the gradient (right panel) and conducted in the absence or constant presence of 0.2% TX-100, respectively. The immunoprecipitates were analyzed by immunoblotting to reveal Lyn and co-immunoprecipitated FcγRII. (C) Second gradient fractionation of mixed fractions 1 and 2 with fractions 4 and 5, obtained from a 0.2% TX-100 density gradient (see the right panel of panel A). Over the mixture, following adjustment to 40% OptiPrep, density gradients were formed without (left panel) or with 0.2% TX-100 (right panel). After ultracentrifugation, the distribution of FcγRII and Lyn in the gradient fractions was examined. (D) Immunoprecipitation of Lyn from fractions 1, 2, 4, and 5 of the gradients presented in panel C. Immunoprecipitates derived form TX-100-devoid and TX-100-containing gradient fractions were washed without or with 0.2% TX-100, respectively. Aside from Lyn, the presence of FcγRII in the precipitates was analyzed. (E) Second gradient fractionations of fractions 4, 5, and 7 obtained from the 0.2% TX-100 density gradient (see the right panel of panel A).

originally associated with DRMs during the detergent extraction of cells but was progressively solubilized and dissociated from DRMs upon centrifugation, when it started to float in detergent-containing density gradients. Lysis of cells under detergent-limiting conditions might favor such insufficient FcγRII solubilization. To assess this problem, various amounts of cells, ranging from 12.5×10^6 to 1.25×10^6, were solubilized in 0.2% TX-100, and soluble and insoluble fractions were separated by high-speed ultracentrifugation. Under these conditions, DRMs were recovered in pellets, as indicated by the presence of Lyn (Figure 2A) and the exclusion of Na^+,K^+-ATPase (not shown). In contrast to Lyn, the content of non-cross-linked FcγRII in pellets decreased as the ratio of TX-100 to the cell amount was increased. When 12.5×10^6 cells were lysed, corresponding to the conditions employed for density gradient fractionation (see Figure 1A), the receptor was insoluble in 0.2% TX-100. This line of data is consistent with the idea that fractionation of cell lysates over TX-100-containing gradients promotes progressive solubilization of the receptor, as floating FcγRII-containing DRMs encounter fresh doses of the detergent. This event could account for a continuous shifting of FcγRII toward higher-density fractions, as the detergent concentrations used in lysis buffers and gradients increased from 0.05 to 0.5% (Figure 1A, right panel).

Lysis of a 10 times smaller amount of cells (1.25×10^6) led to complete solubilization of the non-cross-linked FcγRII (Figure 2A). To further pursue the receptor behavior, the supernatant from a 1.25×10^6 cell lysate isolated by high-speed centrifugation and containing solubilized FcγRII was

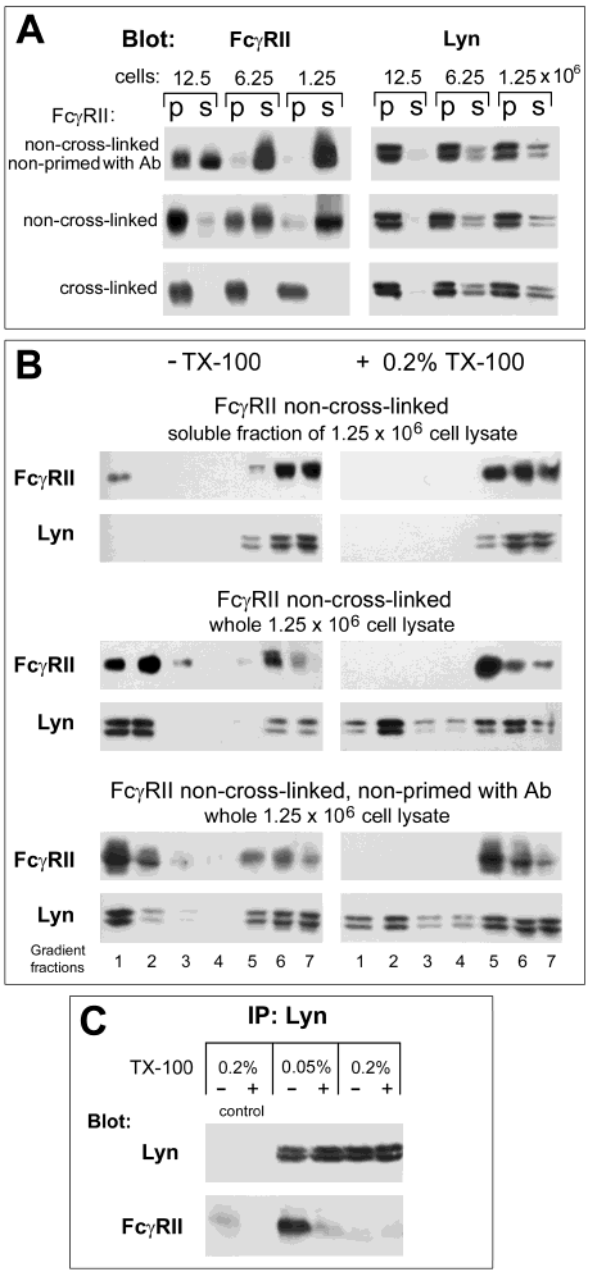

FIGURE 2: Solubility of FcγRII in TX-100. (A) U937 cells ($1.25-12.5 \times 10^6$) were either left untreated with antibody (nonprimed FcγRII), exposed to biotin-labeled mouse anti-FcγRII IgG alone (non-cross-linked receptor), or additionally incubated with anti-mouse IgG to cross-link FcγRII. Subsequently, cells were lysed in 200 μL of 0.2% TX-100. Insoluble (p) and soluble (s) fractions were separated by high-speed centrifugation and analyzed for the presence of FcγRII and Lyn. (B) The top panel shows density gradient fractionation of a TX-100-soluble fraction(s) derived from 1.25×10^6 cells exposed to anti-FcγRII and lysed in 200 μL of 0.2% TX-100 (see the middle panel of panel A). The middle and bottom panels show density gradient fractionation of 0.2% TX-100 lysates (1.25×10^6 cells in 200 μL of lysis buffer) of cells treated with anti-FcγRII (non-cross-linked receptor, middle panel) or not treated with the antibody (bottom panel). The cell lysates were fractionated over detergent-free or 0.2% TX-100-containing density gradients, as indicated, and analyzed for FcγRII and Lyn distribution in the gradient factions. (C) Immunoprecipitation of Lyn from whole cell lysates (6.25×10^6 cells/200 μL of 0.05%−0.2% TX-100) conducted in the absence (−) or constant presence of TX-100 (+). The immunoprecipitates were analyzed by immunoblotting to reveal Lyn and co-immunoprecipitated FcγRII. A control for the immunoprecipitation was obtained by omitting the anti-Lyn antibody.

subjected to a density gradient fractionation. Under these conditions, only minute amounts of non-cross-linked FcγRII floated up to fraction 1 in the absence of TX-100, confirming efficient TX-100 solubility of the output receptor fraction (Figure 2B, top panel). Surprisingly, fractionation of the whole 1.25×10^6 cell lysate over a detergent-free density gradient resulted in translocation of significant amounts of the receptor ($73.1 \pm 1.1\%$, $n = 3$) to DRM fractions 1 and 2, abundant in Lyn (Figure 2B, middle panel). Inclusion of 0.2% TX-100 in the gradient prevented the receptor shift, retarding FcγRII in fractions 5−7 (Figure 2B, middle panel). These results were similar to those obtained after fractionation of 12.5×10^6 cell lysates over detergent-free gradients, shown in Figure 1A (left panel). Taken together, this set of experiments supports the possibility that fractionation of detergent cell lysates over detergent-free gradients enables reconstitution of membrane fragments and association of a distinct subset of solubilized membrane proteins, including FcγRII, with the formed DRMs.

This prediction was further underscored by studies of FcγRII nonprimed with anti-FcγRII antibody. This intact FcγRII exhibited higher detergent solubility than the antibody-primed receptor (Figure 2A). Nonprimed, fully solubilized FcγRII from 0.2% TX-100 cell lysate (1.25×10^6 cells) also associated with DRM fractions 1 and 2, when subjected to fractionation over a density gradient lacking detergent (Figure 2B, bottom panel). As expected, in a density gradient supplemented with 0.2% TX-100 the receptor was recovered in high-density fractions 5−7 (Figure 2B, bottom panel).

The fractionation experiments indicate that non-cross-linked FcγRII displays a low affinity for DRMs. Its behavior during density gradient fractionation was distinct from that of other transmembrane proteins, including TfR and Na^+,K^+-ATPase, which did not associate with DRMs under any fractionation conditions. In contrast, the conditions of density gradient fractionation of detergent cell lysates can either augment or attenuate FcγRII−DRM interactions, making for a difficult conclusion about the receptor partition in native membrane rafts. In another approach to this problem, co-immunoprecipitation of FcγRII and Lyn from whole cell lysates was conducted. The receptor accompanied Lyn only when cells were lysed in 0.05% TX-100 and the detergent was omitted during subsequent washing of the Lyn precipitates. No co-immunoprecipitation was observed at 0.2% TX-100, probably reflecting efficient receptor solubility at the detergent:cell ratio applied in this experiments (Figure 2C).

Association of Cross-Linked FcγRII with DRMs. We next examined the interaction of cross-linked FcγRII with DRMs with the use of the fractionation scheme used above for the non-cross-linked receptor. As expected, after fractionation of 0.05−0.5% TX-100 cell lysates over detergent-free gradients, 79−85% of activated FcγRII was found together with the majority of Lyn in buoyant DRM fractions 1 and 2 (Figure 3A, left panel). Interestingly, the association of activated FcγRII with DRMs was also found when fractionation was carried out over gradients including 0.05−0.5% TX-100: $52.0 \pm 9.7\%$ of the FcγRII at 0.2% TX-100 or $18.1 \pm 4.2\%$ of the receptor at at 0.5% TX-100 ($n = 3$) was still detected in low-density buoyant fractions 1 and 2 of the gradients, which also contained 81−53% of Lyn (Figure 3A, right panel). Accordingly, the cross-linked receptor was found in TX-100-insoluble fractions pelleted during high-

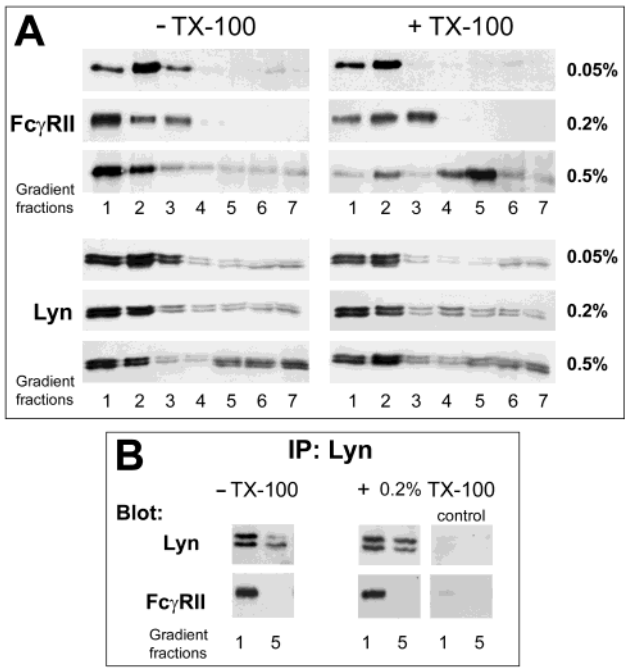

FIGURE 3: Association of cross-linked FcγRII with DRMs revealed in the presence of TX-100. U937 cells (12.5×10^6) were pretreated with biotin-labeled mouse anti-FcγRII followed by anti-mouse IgG (0 °C for 30 min each) to cross-link the receptor. Later, the cells were lysed with 200 μL of TX-100 in the range of 0.05−0.5% and fractionated over OptiPrep density gradients either devoid of TX-100 or supplemented with 0.05−0.5% detergent, as in Figure 1. (A) Immunoblotting analysis of the distribution of cross-linked FcγRII and Lyn in fractions derived from detergent-free gradients (left panel) and gradients containing various concentrations of TX-100 (right panel). On the right are shown TX-100 concentrations used for cell lysis, equal to those added later to the indicated gradients. (B) Fractions 1 and 5 of density gradients without or with 0.2% TX-100 (see panel A) were subjected to immunoprecipitation with rabbit anti-Lyn, in the absence (left panel) or constant presence of 0.2% TX-100 (right panel), respectively. An immunoprecipitation control was obtained by omitting the anti-Lyn antibody. Immunoblotting analysis was performed to examine the efficiency of Lyn immunoprecipitation and co-immunoprecipitation of FcγRII.

speed centrifugation at any detergent:cell ratio examined (Figure 2A). Moreover, large amounts of activated FcγRII were co-immunoprecipitated with Lyn from fraction 1 of the density gradients, independent of the presence of 0.2% TX-100 during fractionation and immunoprecipitation (Figure 3B). In controls, when the anti-Lyn antibody was omitted during the immunoprecipitation, only traces of FcγRII were detected in pellets (Figure 3B). Thus, in contrast to nonactivated FcγRII, the association of cross-linked FcγRII with DRMs is resistant to solubilization in TX-100 at concentrations of >0.2%.

Because the constant presence of TX-100 during DRM isolation had a profound effect on the detection of FcγRII in DRM fractions after density gradient ultracentrifugation, the total protein composition of DRMs was also analyzed. For this purpose, unstimulated and FcγRII-stimulated U937 cells were lysed in 0.2% TX-100 and fractionated by gradient centrifugation with or without 0.2% TX-100. The protein composition of DRMs obtained in fractions 1 and 2 is visualized in Figure 4. DRMs isolated from cells in the presence of the detergent, independent of FcγRII cross-linking, were significantly poorer in proteins when compared

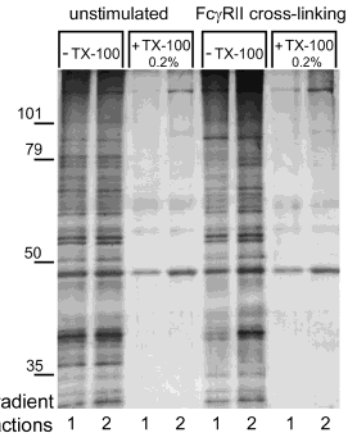

FIGURE 4: Protein composition of DRMs isolated in the absence or presence of 0.2% TX-100 during density gradient centrifugation. U937 cells (12.5×10^6) were lysed in 200 μL of 0.2% TX-100 after pretreatment with mouse anti-FcγRII alone (unstimulated) or after additional incubation with anti-mouse IgG (cross-linked FcγRII). The lysates were fractionated over OptiPrep density gradients either devoid of the detergent or containing 0.2% TX-100. Equal volumes of DRMs in fractions 1 and 2 were loaded onto SDS−PAGE gels, and proteins were visualized by silver staining. At the left, molecular mass standards are shown in kilodaltons.

to DRMs obtained in the absence of TX-100. This difference reflects the possibility of a membrane fragment reconstitution during fractionation of detergent cell lysates over detergent-free gradients as well as possible removal of weakly associated proteins from DRMs in the presence of TX-100 during the lysate fractionation.

The TX-100 Concentration Affects the Presence of TCR in the DRM Fraction. To test whether the presence of TX-100 during gradient centrifugation is essential in studies of the interaction of immunoreceptors with DRMs, we performed further analysis on the TCR of Jurkat cells, since ambiguous data on association of TCR with DRMs were previously reported (*10*, *11*, *18*, *19*). After lysis of unstimulated cells in 0.05−0.2% TX-100 and subsequent fractionation of the lysates over density gradients lacking TX-100, TCR was abundant (80−75% of the total) in low-density fractions 1 and 2 (Figure 5A, left panel). Lck, the tyrosine kinase responsible for TCR phosphorylation, was detected in the same fractions, although small amounts of the kinase were soluble in 0.2% TX-100 and were shifted toward high-density fractions 4−7 (Figure 5A, left panel). The presence of TX-100 during fractionation did not significantly affect the pattern of Lck distribution in the density gradients (Figure 5A, right panel, also Figure 5B). Upon inclusion of 0.05% TX-100 in the gradient, 94.6 ± 8.3% ($n = 3$) of non-cross-linked TCR was still found in fractions 1 and 2; however, in the presence of 0.2% detergent during fractionation, TCR was no longer detected in the DRM fractions (Figure 5A, right panel). Furthermore, in the presence of 0.2% TX-100 during fractionation, only small amounts of cross-linked TCR (6.4 ± 0.8%, $n = 3$) were recovered in the DRM fractions (Figure 5B, right panel). These observations are in agreement with previous reports indicating that the association of cross-linked TCR with DRMs is more sensitive to detergent treatment than the interaction of activated FcγRII (*10*, *11*, *16*). Exclusion of TX-100 from the density gradient led to an enrichment of DRM fractions in the activated TCR (53.6 ± 4.6% of the total TCR, $n = 3$) (Figure 5B, left panel).

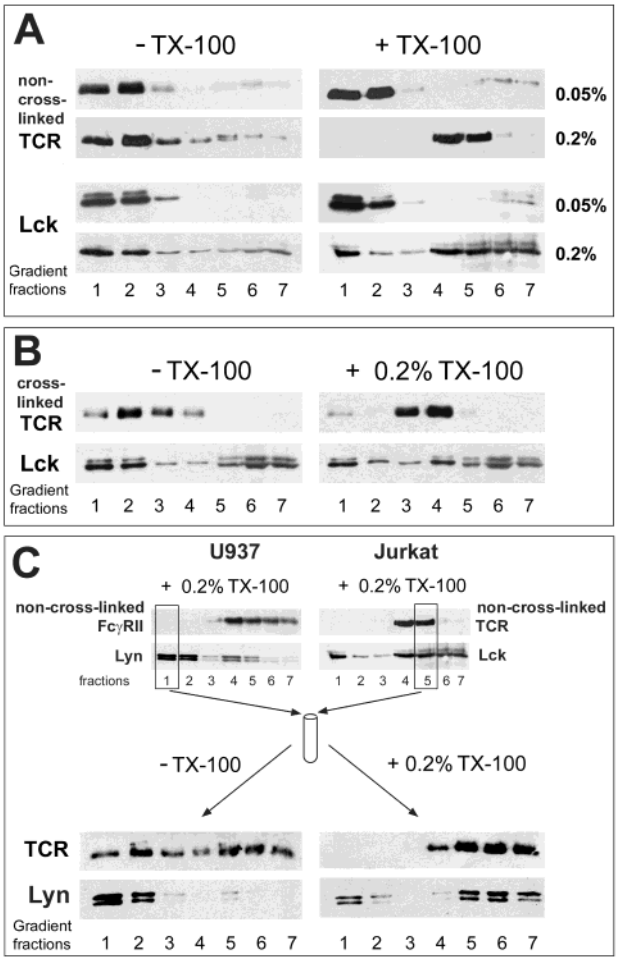

FIGURE 5: TX-100-sensitive association of TCR with DRMs. Jurkat cells (12.5×10^6) were treated either with biotin-labeled mouse anti-CD3ϵ alone (A) or with biotin-labeled mouse anti-CD3ϵ and anti-mouse IgG to cross-link TCR (B) prior to lysis in 200 μL of buffer containing 0.05−0.2% TX-100. (A) The lysates of unstimulated cells were fractionated over OptiPrep density gradients either not supplemented with TX-100 (left panel) or containing the detergent at a concentration corresponding to that used for cell lysis (indicated in the right panel). The distribution of TCR and Lck in gradient fractions is shown. (B) Distribution of cross-linked TCR and Lck in gradient fractions obtained from 0.2% TX-100 cell lysates subjected to fractionation over a detergent-free density gradient (left panel) or a gradient with 0.2% TX-100 (right panel). (C) Second fractionation of mixed gradient fraction 1 derived form unstimulated U937 cells and fraction 5 of unstimulated Jurkat cells. Fraction 1 containing non-cross-linked FcγRII and fraction 5 with non-cross-linked TCR were obtained by centrifugation of 0.2% TX-100 cell lysates over gradients supplemented with 0.2% TX-100. After the solution had been adjusted to 40% OptiPrep, the mixture was subjected to a second fractionation over OptiPrep density gradients formed either without or with 0.2% TX-100. The obtained fractions were examined by immunoblotting for the presence of TCR and Lyn kinase.

Finally, we mixed fraction 5 of the 0.2% TX-100 gradient, containing non-cross-linked, TX-100-soluble TCR and Lck of Jurkat cells, with fraction 1 of the 0.2% TX-100 gradient obtained after ultracentrifugation of unstimulated U937 cells (Figure 5C). The U937-delivered fraction contained DRMs enriched in Lyn, but it lacked FcγRII (Figure 5C, left panel). Surprisingly, refractionation of the mixture over a detergent-free gradient revealed that $31.6 \pm 5.6\%$ ($n = 3$) of the TCR was able to associate with exogenous DRMs and was now recovered in buoyant fractions 1 and 2 of the gradient, together with Lyn of U937 cells (Figure 5C, left panel). However, the presence of 0.2% TX-100 during the gradient fractionation of the mixture prevented the association of non-cross-linked TCR with DRMs (Figure 5C, right panel).

Concentration of TX-100 in Density Gradient Fractions. The strong influence of TX-100 during cell fractionation, exerted on the association of immunoreceptors with DRMs, prompted us to estimate the detergent concentration in density gradient fractions. It was found that fractionation of 0.2% TX-100 cell lysates over a detergent-free OptiPrep density gradient let to a significant dilution of the detergent. As a result, the concentration of TX-100 at the top of the gradient was reduced to 0.010−0.013%, below the critical micelle concentration [Figure 6A (▲)]. On the other hand, when the gradient fractionation was carried out in the presence of 0.2% TX-100, the concentration of the detergent in fractions 1−3 was increased to 0.27% with simultaneous depletion of the detergent in high-density fractions 5−7 [Figure 6A (●)].

The TX-100 concentration was also estimated after fractionation of the 0.2% TX-100 cell lysates in a sucrose density gradient, as is most often used for DRM isolation. The pattern of TX-100 distribution in both gradients, either containing TX-100 or not supplemented with the detergent, resembled the profiles of the detergent distribution in OptiPrep gradients. However, TX-100 concentrations in low-density fractions 1−3 derived from the sucrose density gradient formed without the detergent were as low as 0.003−0.01% [Figure 6B (▲)]. Fractions 1−3 derived from the sucrose gradient containing TX-100 were significantly enriched with the detergent, reaching 0.31% [Figure 6B (●)]. Ultracentrifugation of the sucrose density gradient containing 0.2% TX-100, but devoid of cell lysates, also shifted the detergent toward the top of the gradient. However, the detergent was distributed in the gradient in a more linear fashion [Figure 6B (○)]. The TX-100 concentration reached 0.26% in fractions 1 and 2 and 0.21% in fraction 3, which was lower than the concentration achieved during fractionation of TX-100 cell lysates. This suggests that accumulation of TX-100 in low-density gradient fractions 1−3 could result from flotation of free detergent micelles and detergent micelles containing membrane lipids and scarce proteins.

On the other hand, the S-shaped, nonlinear profile of the TX-100 distribution in gradients acquired during fractionation of cell lysates may indicate that mixed protein/TX-100 or lipid/TX-100 micelles behave on gradients in a manner different from that of pure TX-100 micelles. To test this possibility, we examined the distribution of TX-100 in OptiPrep density gradients after fractionation of PC/TX-100 mixtures at PC:TX-100 molar ratios of 1:5 and 1:10. However, no significant influence of PC on the linear profile of TX-100 distribution in gradient fractions was detected except for a small but repeated enrichment of the detergent in fraction 4 of the gradient at a PC:TX-100 ratio of 1:5 (Figure 6C). Fraction 4 also contained a vast majority of the PC loaded on the gradient [Figure 6C (− − −)]. With high doses of PC (PC:TX-100 molar ratio of >1:6), the TX-100 content in samples can be overestimated (Figure 6D), which seems to account for the increased level of TX-100 detected in fraction 4 of the gradient supplemented with PC. However, this simplified experimental system does not provide an explanation for accumulation of TX-100 in low-density fractions 1−3 with concomitant depletion of the

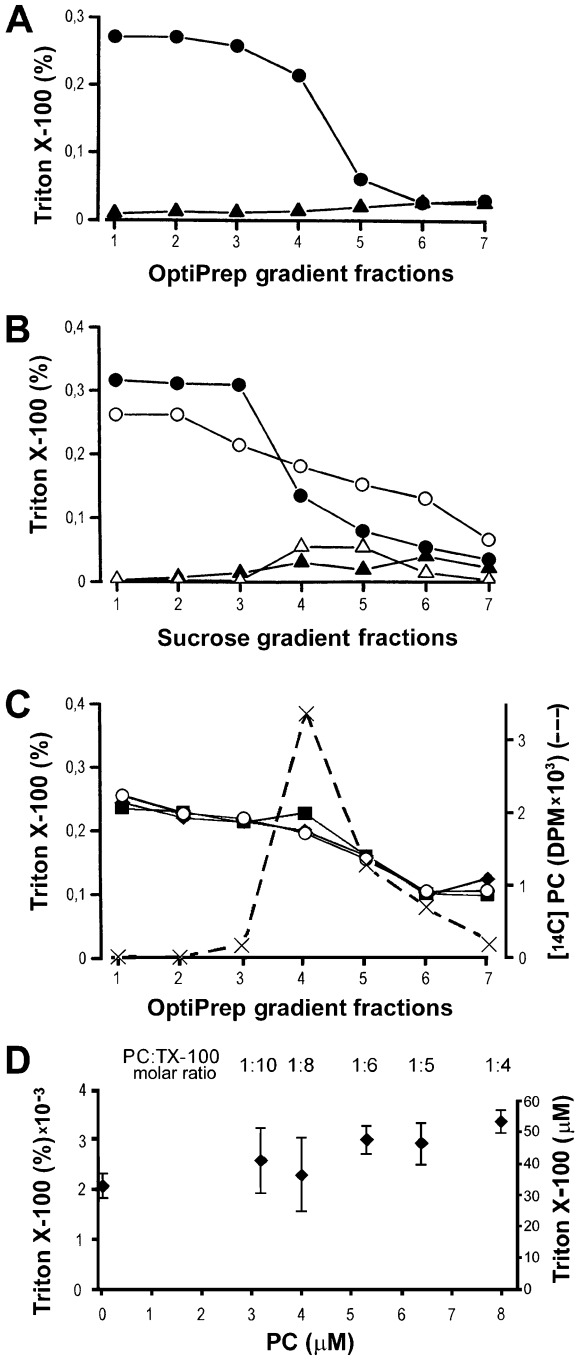

FIGURE 6: Distribution of TX-100 in OptiPrep and in sucrose density gradients. (A) Concentration of TX-100 in OptiPrep density gradient fractions obtained after centrifugation of 0.2% TX-100 cell lysates in a density gradient devoid of (▲) or supplemented with 0.2% TX-100 (●). (B) Concentration of TX-100 in sucrose density gradient fractions derived from gradients not supplemented with the detergent (▲ and △) or containing 0.2% TX-100 (● and ○). Filled symbols indicate TX-100 profiles after fractionation of 0.2% TX-100 cell lysates; empty symbols show the TX-100 concentration in fractions obtained after ultracentrifugation of sucrose gradients without cell lysates. (C) Distribution of TX-100 in 0.2% TX-100 OptiPrep gradients after fractionation of PC/TX-100 micelles [1:5 molar ratio (■) and 1:10 molar ratio (◆)] or TX-100 micelles alone (○). The input samples contained 200 μL of 0.2% TX-100 (≈3.2 mM) without or with 0.64 mM PC or 0.32 mM PC. The PC samples contained 0.025 μCi of [^{14}C]PC (6000 dpm). (D) Influence of PC on the estimation of the amount of TX-100. A constant amount of TX-100 (0.002%, corresponding to 32 μM) was mixed with increasing amounts of PC, and the TX-100 concentration was estimated using the phosphomolybdate method described in Materials and Methods.

detergent in high-density fractions 5−7 occurring during density gradient fractionation of cell lysates.

DISCUSSION

Since the first reports about the TX-100 insolubility of membrane fragments enriched in cholesterol and glycolipids and containing GPI-anchored proteins as well as Src family tyrosine kinases (*17*, *25*), a great effort has been undertaken to elucidate this phenomenon. Studies of model membranes revealed that the insolubility results from tight packing of acyl chains of sphingolipids, phospholipids, and cholesterol, together acquiring a liquid ordered state (*26*). DRMs isolated from cells displayed a similar lipid composition, supporting the idea that liquid ordered phase rafts exist in the plasma membrane (*2*, *27*, *28*). Isolation of detergent-insoluble membrane fragments became a commonly used method for studying proteins that are either constitutively located or transiently associated with the lipid rafts. On the basis of these studies, rafts have emerged as signaling platforms where activated immunoreceptors are recruited and undergo phosphorylation by raft-residing tyrosine kinases of the Src family (*29*). It is interesting, however, that in the case of FcϵRI, very low TX-100 concentrations were used for cell lysis (0.03−0.05%) to demonstrate the association of the cross-linked receptor with DRMs (*30*). In addition, it should be underlined that, in the majority of studies concerning the interaction of immunoreceptors with rafts, isolation of DRMs from TX-100 cell lysates was performed over detergent-free density gradients (*9*−*11*, *14*, *17*, *19*).

The results presented in this study show that the presence of TX-100 during gradient fractionation has a profound effect on the location of FcγRII and TCR in DRM fractions. The effect was especially prominent when non-cross-linked receptors were analyzed; depending on the conditions of fractionation, the receptors were recovered either in DRM fractions or in higher-density fractions. After cross-linking had been carried out, FcγRII and TCR were associated with DRMs independent of the presence of TX-100 during gradient fractionation, although inclusion of the detergent decreased the amounts of the receptors found in DRMs by up to 18.1% of FcγRII and 6.4% of TCR at 0.5 and 0.2% TX-100, respectively. These findings can be explained by two phenomena apparently occurring during density gradient fractionation of detergent cell lysates over detergent-free and detergent-containing gradients. In the former case, the concentration of TX-100 present in the cell lysates and loaded on the gradient is significantly reduced as the fractionation proceeds (Figure 6). It is noteworthy that the removal of detergents from membrane samples solubilized in nonionic detergents is a frequently used strategy for membrane reconstitution (*31*−*33*). Among others, sedimentation of components of a Nonidet P-40-solubilized ciliary membrane in a detergent-free sucrose gradient was shown to facilitate reconstitution of the protein/lipid composition of the membrane (*34*). Therefore, the procedure of DRM isolation from TX-100 cell lysates over detergent-free gradients seems to mimic the conditions required for membrane reconstitution leading to the enrichment of DRMs in some TX-100-soluble membrane proteins. This suggestion is supported by our results, indicating that refractionation of a mixture of DRM fractions (devoid of non-cross-linked FcγRII) and high-density fractions (containing the receptor) over a detergent-

free OptiPrep density gradient led to the appearance of FcγRII in DRM fractions (Figure 1C) and the association of the receptor with DRM-residing Lyn, as shown by co-immunoprecipitation studies (Figure 1D). This event took place even when DRMs derived from U937 cells were mixed with Jurkat-delivered TX-100-soluble TCR (Figure 5). These data indicate that fractionation of the detergent lysates over detergent-free gradients can evoke the association of some of the membrane proteins with DRMs.

On the other hand, close examination of a pattern of migration of the non-cross-linked FcγRII in density gradients containing 0.05−0.5% TX-100 revealed a striking difference between the behavior of the receptor and that of TfR and Na^+,K^+-ATPase, two other transmembrane proteins (Figure 1A, right panel). FcγRII underwent a progressive shift toward higher-density gradient fractions as the detergent concentrations used for cell lysis and gradient preparation increased. This event could imply that non-cross-linked FcγRII remained associated with DRMs during cell lysis, plausibly due to an insufficient solubilization, but dissociated from DRMs later, upon density gradient fractionation under the influence of newly encountered doses of TX-100. The detergent could extract the receptor from DRMs into micelles which were retarded in the gradient at the equilibrium position, where they were formed. Accordingly, studies of TX-100 solubility of FcγRII confirmed that a proper detergent:cell ratio used for cell lysis strongly affected the efficiency of the non-cross-linked receptor solubilization (Figure 2A). Under conditions of insufficient solubilization of FcγRII upon cell lysis, the presence of TX-100 during density gradient fractionation can promote further solubilization of FcγRII. This receptor solubilization seems to be further enhanced due to flotation of TX-100 micelles in TX-100-supplemented gradients, leading to a detergent enrichment in buoyant fractions (Figure 6). Therefore, fractionation of detergent cell lysates over detergent-containing gradients enables isolation of DRMs devoid of weakly associated proteins, like non-cross-linked FcγRII and TCR.

It is interesting to note that density gradient fractionation of cell lysates obtained under conditions ensuring the full solubility of the non-cross-linked FcγRII (1.25×10^6 cells in 200 μL of 0.2% TX-100) over a detergent-free gradient resulted in recovery of nearly 80% of the receptor in buoyant DRM fractions 1 and 2, whereas use of a 0.2% TX-100-containing gradient completely abrogated receptor flotation to the low-density fractions (Figure 2B, middle and bottom panels). These studies provide direct evidence for facilitated association of primarily TX-100-solubilized FcγRII with DRMs when the detergent concentration decreased in the course of DRM isolation in detergent-free gradients.

The effects of the absence and presence of TX-100 during density gradient fractionation on DRM protein composition are understandable in view of the results of the analysis of TX-100 concentration in the gradients (Figure 6). During fractionation of the 0.2% TX-100 cell lysates over a detergent-free OptiPrep density gradient, the concentration of TX-100 in low-density fractions reached 0.010−0.013%, being even lower in sucrose density gradients. These values are below the TX-100 critical micelle concentration (0.014−0.015% corresponding to 0.22−0.24 mM; *35*), favoring a reconstitution of membrane fragments and association of DRMs with a distinct subset of membrane proteins, including FcγRII and TCR. In contrast, adding 0.2% TX-100 to the gradients led to flotation and accumulation of the detergent in DRM fractions, abrogating weak interactions between DRMs and these proteins. This indicates that, in both cases, the TX-100 concentration in DRM fractions varies from that used for cell lysis, which has implications for the presence of immunoreceptors (and other proteins; see Figure 4) in DRMs and adds a new parameter to the interpretation of results.

We never recovered TfR and Na^+,K^+-ATPase in DRM fractions regardless of the presence or absence of TX-100 during gradient fractionation. Therefore, we cannot exclude the possibility that isolation of DRMs in TX-100-devoid gradients reveals membrane proteins that in native membranes are weakly associated with rafts. Non-cross-linked immunoreceptors can belong to this group of proteins. The interactions of such proteins with raft lipids can be weaker than the interactions of these proteins with detergents, making them susceptible to solubilization. Only proteins that are expected to pack tightly into the ordered lipid environment of rafts, like the Src family kinases and GPI-anchored proteins, are resistant to harsh detergent treatment (this paper and refs *2* and *36*). Nevertheless, employing very low TX-100 concentrations for DRM isolation has further limitations. At a low detergent:cell ratio, membranes are not completely solubilized and mixed bilayers that incorporate detergent can be formed instead of an assembly of micelles of detergent and detergent-soluble membrane components (*34*, *37*). Accordingly, at 0.05% TX-100, non-cross-liked FcγRII was detected in DRM fractions even in the presence of TX-100 during gradient centrifugation. Taken together, the various conditions used for DRM isolation can account for discrepancies in the data concerning the presence of immunoreceptors and their downstream signaling molecules in rafts (*9*−*11*, *18*−*20*).

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