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The Transferrin Receptor and the Tetraspanin Web Molecules CD9, CD81, and CD9P-1 Are Differentially Sorted Into Exosomes After TPA Treatment of K562 Cells

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Abstract Here we show that treatment of K562 cells with the phorbol ester TPA induces the down-modulation of various surface antigens. Among them, the transferrin receptor (TfR), the tetraspanin CD81, and a CD81-associated protein, CD9P-1, were unique in that their expression levels were lower after 24 h incubation than after 3 h. We demonstrated that like the TfR, CD81 was internalized at early times, and was less synthesized at latter times. Despite the association of a fraction of the TfR with CD81, these two molecules were subjected to different fates. TPA increased targeting of CD81 and CD9P-1 into exosomes but strongly reduced the localization of the TfR in these vesicles. Using this model we have shown that a fraction of CD81 and CD9P-1 in exosomes comes from a surface pool and that these molecules remain associated in exosomes. However, CD9P-1 could be targeted to exosomes in the absence of CD81 and of another tetraspanin, CD9. The targeting of CD9 into exosomes did not require palmitoylation of the protein. J. Cell. Biochem. 102: 650–664, 2007.

Key words: tetraspanins; exosomes; CD81

Tetraspanins are molecules with 4-transmembrane domains that are expressed in all cell types. Specific sequence conservation and a specific fold in the second extracellular region help to distinguish these molecules from other molecules with 4-transmembrane domains. Tetraspanins have been implicated in various biological processes such as cell adhesion, migration, cell fusion, co-stimulation, signal transduction, and differentiation (reviewed in Boucheix and Rubinstein, 2001; Hemler, 2003; Levy and Shoham, 2005). Although their precise function remains unknown, they are believed to organize new types of microdomains, different from rafts [Boucheix and Rubinstein, 2001; Hemler, 2003; Levy and Shoham, 2005]. Several tetraspanins associate directly with a limited number of specific partner molecules, which they may connect to these microdomains [Charrin et al., 2003b]. For example laminin-binding integrins ($\alpha3\beta1$, $\alpha6\beta1$, $\alpha7\beta1$, $\alpha6\beta4$) are partners for CD151 [Yauch et al., 1998; Serru et al., 1999; Sterk et al., 2000], while two partners of CD9 and CD81 are newly identified proteins with Ig domains called CD9P-1/EWI-F (CD315) and EWI-2 (CD316) [Charrin et al., 2001; Clark et al., 2001; Stipp et al., 2001a,b]. Tetraspanin-to-tetraspanin interactions are likely to be crucial for the formation of the microdomains, and have been shown to rely at least in part on the palmitoylation of the proteins and on an interaction with cholesterol [Charrin et al., 2003b,c].

If tetraspanins are present at cell surface, some of them are also localized in intracellular compartments. A study of the expression of

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tetraspanins on the megakaryoblastic cell line Mo7e showed that the intracellular localization of tetraspanins CD63 and CD151 was predominant as compared to tetraspanins CD9 and CD81 [Fitter et al., 1999]. In this regard CD63 is highly enriched in late endosomes, lysosomes, and multivesicular bodies (MVB) which also contain several other tetraspanins such as CD81, CD53, and CD82 [Escola et al., 1998]. Consistent with the expression of these molecules in MVB internal vesicles, tetraspanins are also highly enriched on exosomes [Escola et al., 1998].

Exosomes are small 50–100 nm vesicles produced by many cell types including reticulocytes [Johnstone et al., 1987], platelets [Heijnen et al., 1999], B and T lymphoid cells [Raposo et al., 1996; Blanchard et al., 2002; Fritzsching et al., 2002], dendritic cells [Thery et al., 1999], as well as epithelials cells [van Niel et al., 2001]. These vesicles originate from exocytosis of vesicles contained by MVB, which are formed by inward budding from the limiting membrane into the lumen of endosomes [Stoorvogel et al., 2002; Thery et al., 2002b]. Exosomes have been shown to mediate antigen presentation [Zitvogel et al., 1998] and also to transfer material from one cell to another [Fritzsching et al., 2002; Thery et al., 2002a]. Exosome release plays an important role in the maturation of reticulocytes by contributing to the clearance of obsolete proteins [Blanc et al., 2005]. Some retroviruses and in particular HIV have the ability to hijack the intracellular machinery of MVB biogenesis for budding at cell surface in lymphoid T cells [Pelchen-Matthews et al., 2003]. Additionally, in macrophages HIV accumulates and buds mainly in MVB [Raposo et al., 2002; Pelchen-Matthews et al., 2003]. Accordingly, HIV particles produced by macrophages express several tetraspanins, including CD63 [Pelchen-Matthews et al., 2003], and anti-CD63 mAb inhibits infection by macrophage strains [von Lindern et al., 2003].

How proteins are targeted to MVB and exosomes is poorly understood. MVBs receive biosynthetic cargo from the *trans*-Golgi network (TGN), as well as molecules that have been internalized by way of endocytosis [Stern et al., 2006]. The transferrin receptor (TfR) molecules present on exosomes produced during reticulocyte maturation originate from a surface pool that was internalized. The targeting of TfR to exosomes was suggested to involve a sequence encompassing the YxxΦ (where Φ is a hydrophobic amino acid and x any amino acid) internalization motif which interacts with the heat shock protein hsc70 and Alix, a component of the machinery for sorting proteins in the intralumenal vesicles of MVB [De Gassart et al., 2004]. In contrast, in antigen-presenting cells, MHC class II molecules are transported through the Golgi apparatus to organelles of the endocytic pathway, including MVB, collectively referred as to MHC class II compartments MIICs [Geuze, 1998]. Additionally, the C-terminal cytosolic tail of the tetraspanin CD63 ends with a GYxxΦ motif that interacts with several adaptor protein complexes and is critical to the sorting into late endosomes/lysosomes [Bonifacino and Traub, 2003]. A substantial fraction of CD63 passes through the plasma membrane before reaching lysosomes [Janvier and Bonifacino, 2005], but other data suggest that a fraction of CD63 may be directly transported from the TGN to late endosomes, [Rous et al., 2002; Janvier and Bonifacino, 2005]. Most tetraspanins, including CD9, CD81, and many exosomes proteins do not have a YxxΦ motif, indicating that several mechanisms are used for sorting proteins into exosomes. In this regard lipid rafts-resident proteins were recently identified in exosomes secreted by lymphoid B cells and K562 cells [De Gassart et al., 2003]. Additionally, the lipid composition of exosomes resembles that of rafts, and exosomes contain detergent resistant membranes [Wubbolts et al., 2003]. These data led to the suggestion that rafts may contribute to proteins sorting into exosomes [De Gassart et al., 2004; Wubbolts et al., 2003]. Palmitoylation allows the targeting of cytoplasmic and transmembrane proteins to rafts [Resh, 1999], but it is not known whether this post-translational modification contributes to exosome targeting.

In this study, we have developed a model where the targeting of CD81 and other tetraspanins into exosomes can be synchronously augmented. We have used this model to further characterize the sorting of CD81 and its molecular partner CD9P-1 into exosomes.

MATERIALS AND METHODS

Monoclonal Antibodies, Indirect Immunofluorescence, and Reagents

The anti-tetraspanins mAbs used in this study have been previously described [Charrin

et al., 2001, 2003a]: SYB-1 (CD9), TS81 (CD81), TS53 (CD53), TS63 (CD63), TS82, TS82b (CD82), and TS151 (CD151). Other anti-tetraspanin mAbs were M38, Z81 (CD81, [Imai and Yoshie, 1993; Azorsa et al., 1999]), and 11B1G4 (CD151, [Sincock et al., 1999]). The mAb 1F11 (CD9P-1), 12A12 (CD55), and V5-vjf (integrin α5 subunit) have been produced in our laboratory [Lozahic et al., 2000; Charrin et al., 2001, 2003a]. The anti-TfR mAb 12B11 was produced in our laboratory and was used for immunoprecipitations. The anti-TfR mAb B-G24 from Diaclone (Besançon, France) was used for cytometry analysis and the anti-TfR mAb H68.4 (Zymed, San Francisco, CA) was used for Western-blot and confocal microscopy. Other mAb were anti-Annexin 2 (BD Transduction Laboratories, Lexington, KY), anti-Tsg101 (Genetex, San-Antonio, TX), and 1B5 (anti-HSC-70, Stressgen, Victoria, BC Canada). Flow-cytometry analysis was performed as previously described [Rubinstein et al., 1996], after labeling intact cells at 4°C with the appropriate mAb. TPA (12-*O*-Tetradecanoylphorbol 13-Acetate) was from Sigma (St. Louis, MO) and was used at 10 ng/ml. The PKC inhibitor GF109203X and the tyrosine kinase inhibitor herbimycin A were from Calbiochem (La Jolla) and used at 2 μM.

Isolation of Exosomes

Exosomes were isolated by differential centrifugation as described previously [Raposo et al., 1996; Escola et al., 1998]. K562 cells were washed in RPMI, resuspended at the concentration of 10^6 cells/ml in the same medium and incubated for 20–24 h, in the presence or absence of 10 ng/ml TPA. The cells were centrifuged for 10 min at 200*g* (pellet P1) and the supernatant was removed and centrifuged twice for 10 min at 600*g*. Supernatants were then sequentially centrifuged at 2,000*g* twice for 15 min, once at 10,000*g* for 30 min and once at 100,000*g* for 60 min (yielding pellet P5), using a SW41 rotor (Beckman Instruments, Inc., Fullerton, CA). P1 contained the cells, whereas P5 was enriched in exosomes. The cells (P1) were lysed at the concentration of 2×10^7/ml in a buffer containing 1% Triton X-100 in lysis buffer (see below) before addition of concentrated Laemmli buffer in the post-nuclear supernatant. P5 was directly solubilized in Laemmli sample buffer without reducing agents. For the P1 fraction, an extract corresponding to ~266,000 cells was analyzed. For the P5 fraction, each lane was loaded with the pellet obtained from $\sim 10^7$ cells.

Metabolic Labeling, Biotin-Labeling, and Immunoprecipitation

For metabolic labeling, K562 cells treated or not with TPA for 20 h were washed twice in RPMI medium and incubated for 30 min at 37°C in cysteine and methionine-deficient medium at the concentration of 15×10^6 cells/ml. [^{35}S] Methionine and [^{35}S] cysteine (expre^{35}S^{35}S, Perkin Elmer Life Sciences, Zaventem, Belgium) were then added for 30 min (final concentration 200 μCi/ml) before washing with ice-cold PBS. The cells were chased in complete medium and lysed in a buffer containing 1% Triton X-100 (Roche Molecular Biochemicals, Meylan, France) in lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, and protease inhibitors).

For surface biotin-labeling, the cells were washed three times in PBS and incubated in PBS containing 0.5 mg/ml EZ-link-Sulfo-NHS-LC-biotin. After 30 min incubation at 4°C, the cells were washed three times in PBS and resuspended in RPMI medium in the presence or absence of 10 ng/ml TPA. After 20 h treatment, the cells and exosome fractions were lysed in lysis buffer supplemented with 1% Brij 97 (Sigma) and 1mM $CaCl_2$ and 1mM $MgCl_2$. Immunoprecipitations and Western-blot were performed as previously described [Charrin et al., 2001].

RNA Interference

The cells were washed twice in RPMI and resuspended at a concentration of 2.5×10^7 cells/ml. Four hundred microliters of the cell suspension was electroporated (300V, 500 μF) using the GenePulser apparatus (Biorad, Hercules, CA) in the presence of 0.2 nmol siRNA specific to CD81 or a control siRNA. After 24 h, a second electroporation was performed using a CD9 siRNA. TPA was added 24 h after the second transfection. The sequences targeted by RNAi were GAG CAT CTT CGA GCA AGA A (CD9) and CAC GTC GCC TTC AAC TGT A (CD81).

Confocal Microscopy

K562 cells treated or not with TPA were seeded on poly-lysine coated slides, before fixation for 20 min in acetone at −20°C. After drying at room temperature, the cells were

incubated 10 min in PBS containing 10% heat-inactivated goat serum and then with a combination of mAb TS81 (IgG2a) and either TS63 or H68.4 (both IgG1) in the same buffer for 20 min at room temperature in a moist chamber. The cells were then washed in PBS and further incubated for 20 min with a combination of goat anti-mouse IgG2a and IgG1, respectively labeled with Alexa 488 and Alexa 568 dyes. After three washes the samples were mounted in mowiol. Analysis was performed with a TCS SP2 confocal microscope (Leica, Wetzlar, Germany).

Endocytosis Assays

To test for the endocytosis of CD81, cells were incubated for 30 min at 4°C in the presence of 10 μg/ml TS81 mAb, washed and incubated at 37°C in RPMI supplemented or not with TPA for the indicated time. For confocal microscopy analysis, they were then treated as above except that the mAb TS81 was omitted during the labeling step. For quantification by flow-cytometry, the TS81 mAb was Alexa 488-labeled, thus providing a relative measure of total cell-associated TS81 mAb and the fraction of TS81 remaining at cell surface was stained at 4°C using a phycoerythrin-labeled goat anti-mouse polyclonal antibody. Analysis was performed using a FacScan cytometer (BD Biosciences) using appropriate compensation settings. The percentage of endocytosis was calculated as follows: $100 \times [1-(FL2_n \times FL1_0)/(FL2_0 \times FL1_n)]$, where $FL1_0$ or $FL1_n$ and $FL2_0$ or $FL2_n$ are the Alexa 488 and phycoerythrin mean fluorescence intensities measured in the FL1 and FL2 detectors, respectively (after subtraction of the value obtained with non-labeled cells) at time 0 or at each time point.

Electron Microscopy

Pellets of exosomes were fixed in PBS containing 2% paraformaldehyde and loaded on formvar/carbon-coated grids. The samples were then postfixed in 1% gluteraldehyde, negatively stained or contrasted in 2% methylcellulose/0.4% uranyl acetate, pH4, and observed in a Zeiss EM 902 electron microscope at 80 kV.

RESULTS

Expression of Various Surface Molecules During Treatment of K562 Cells by TPA

We first studied by immunofluorescence and flow-cytometry the expression levels of tetraspanins, TfR, and other surface molecules after 3 or 24 h treatment with TPA (Fig. 1A). Among the molecules tested here, CD9 and CD82 were strongly upregulated at the surface of K562 cells after 24 h TPA treatment. The surface expression of CD63 did not change, while the expression of CD151 and CD53 dropped rapidly by 40% and 50%, respectively. Not all surface molecules were subjected to changes in expression as there was little change in CD55 expression and the increase in integrin α5β1 expression was late and moderate. CD81, CD9P-1, and the TfR were unique among the molecules tested here as their expression levels were lower after 24 h TPA treatment than after 3 h treatment. For example, the level of CD81 expression dropped to 25–40% of initial level after 24 h treatment, according to the experiments. The strong diminution of TfR expression upon TPA treatment was previously reported [Kohno et al., 1986].

The expression of some of these molecules was examined more frequently during the first hours of treatment (Fig. 1B). The expression levels of all molecules tested (CD81, CD9, CD9P-1, TfR, and the integrin α5) were subjected to a similar initial diminution of expression, which reached 25–50% after 2 h of treatment with TPA. After 3 h, the expression levels of CD9 and of the integrin α5 were restored to the initial levels and then continued to increase. The expression levels of CD81, CD9P-1, and TfR continued to decrease but at a lower rate.

TPA is a known activator of PKC, but it may activate other pathways as well [Rambaratsingh et al., 2003]. The PKC inhibitor GF109203X (Fig. 1C) inhibited the down-modulation of CD9P-1 and CD81 induced by TPA. Thus, PKC actually plays a role in this phenomenon.

TPA-induced CD81 Down-Modulation Is Due to Internalization Followed by Reduced Synthesis

TPA has a dual effect on TfR: it increases the rate of internalization and shuts down its synthesis at later time points [Schonhorn et al., 1995]. The similar outcome of CD81 and TfR expression levels led us to characterize the mechanisms by which CD81 expression was down-modulated.

The initial down-modulation of CD81 is the consequence of internalization, as determined by immunofluorescence on permeabilized cells and confocal microscopy. In unstimulated cells

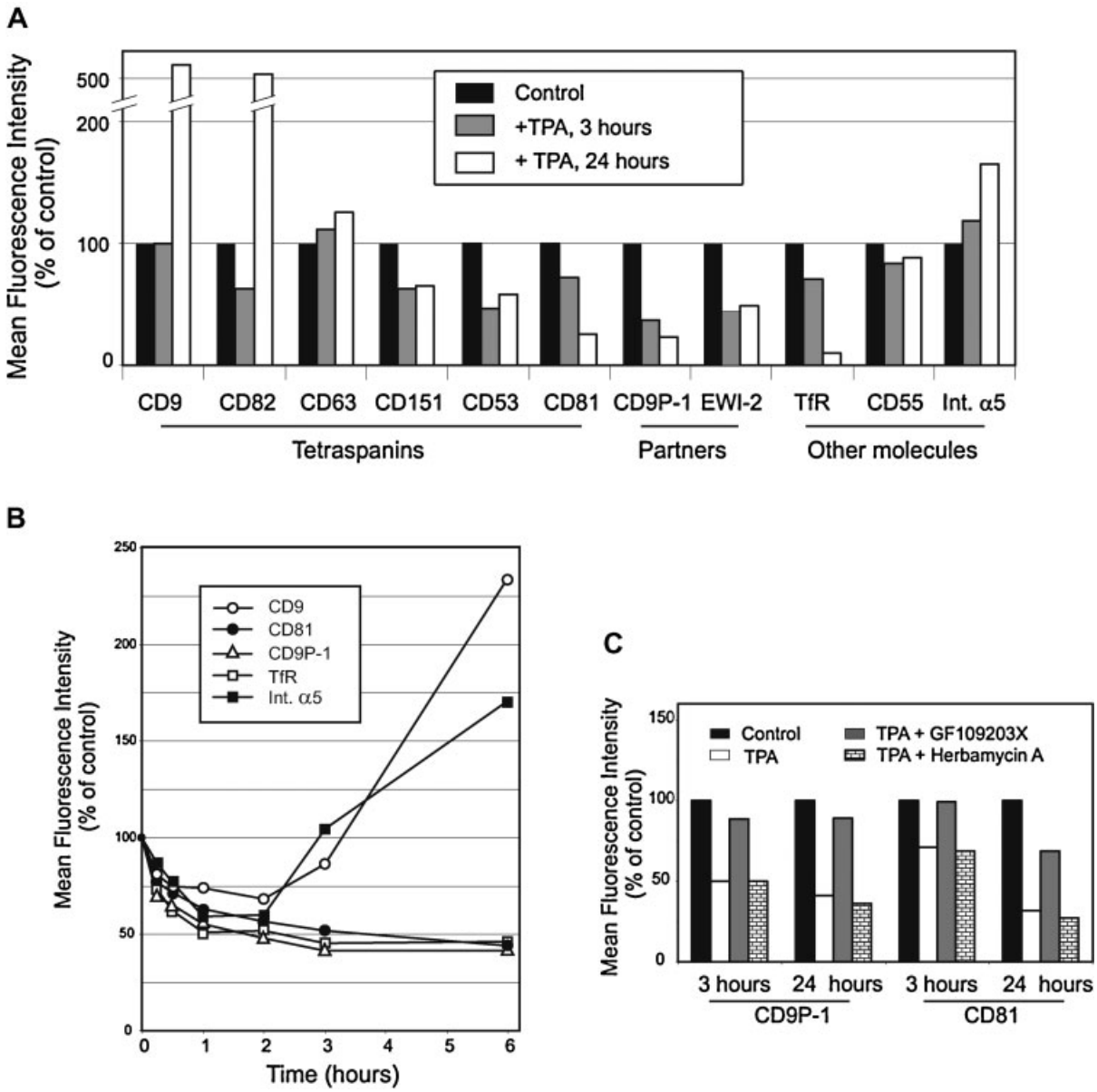

Fig. 1. Expression of tetraspanins during TPA treatment of K562 cells. K562 cells treated or not with TPA for the indicated time were analyzed by indirect immunofluorescence and flow-cytometry for the expression of various surface molecules. The results are shown as the expression level expressed as a percent of control cells. **A**: Expression of the indicated molecules, without TPA treatment, or after 3 h or 24 h treatment. **B**: The expression level of integrin α5, TfR, CD9, CD81, and CD9P-1 was analyzed more frequently during the first 6 h of treatment. **C**: Effect of the PKC inhibitor GF109203X (2 μM) and the tyrosine kinase inhibitor herbimycin A (2 μM) on the down-modulation of CD81 and CD9P-1 expression induced by TPA.

the labeling of CD81 was mainly at the membrane while as expected CD63 was for the most part intracellular (Fig. 2A). A small fraction of CD81 was however intracellular and co-localized with CD63. Reciprocally, a fraction of CD63 was at the cell surface, but is not visible at the exposure used in Figure 2A. After 1 h TPA treatment, the fraction of CD81 at the membrane was diminished while the intracellular fraction strongly increased. The intracellular CD81 labeling had a tendency to concentrate over time, while in contrast the CD63 labeling got dispersed in the cell (Fig. 2A, 6 h). After 6 h of treatment, the major fraction of intracellular CD81 co-localized with CD63. As previously described, the bulk of TfR was intracellular in K562 cells [Schonhorn et al., 1995]. There was little co-localization of this receptor with CD81, both in resting and TPA-treated cells (Fig. 2B).

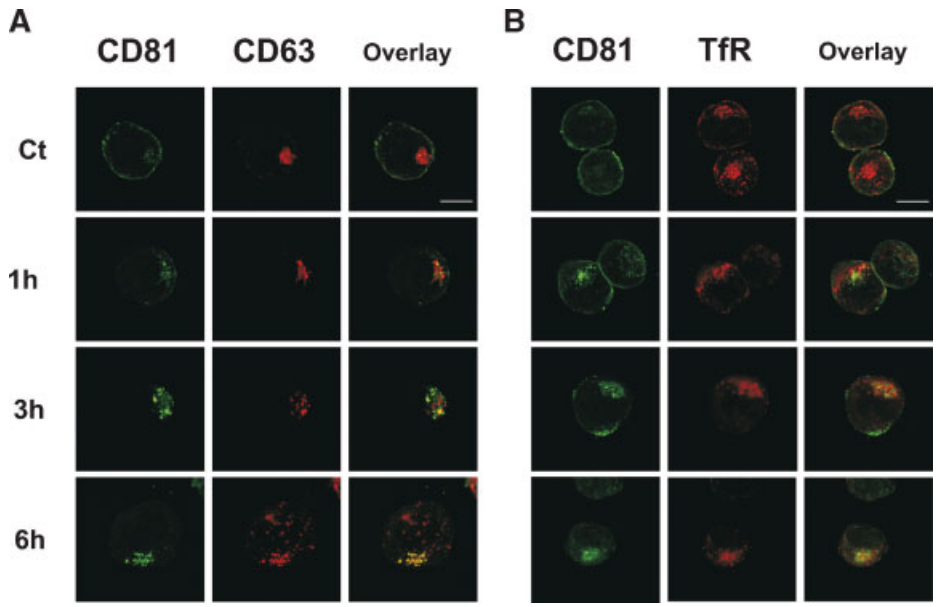

Fig. 2. Change in CD81 cellular distribution following TPA treatment of K562 cells. K562 cells treated or not with TPA for the indicated time were attached to polylysine-coated coverslips, fixed with acetone and labeled with CD81 (green) and either CD63 (red, **A**) or anti-TfR (red, **B**) mAb. The localizations of CD63 and TfR are mainly intracellular, while in control cells CD81 is mainly localized at the plasma membrane. A large fraction of CD81 becomes localized in intracellular compartments after TPA treatment. All images were acquired using the same settings. Bar 10 μm.

To determine whether TPA could affect internalization of CD81, cells were treated with the CD81 mAb and then incubated for various time at 37°C before confocal microscopy analysis. As shown in Figure 3A, a substantial fraction of the TS81 mAb was intracellular and co-localized with CD63 after 6 h of treatment, whether cells were treated or not with TPA. At earlier time points, the internalization of the CD81 mAb was more evident in TPA-treated cells than in non-treated cells. The rate of internalization of CD81 was quantified by flow-cytometry, as described in Material and Methods section. As expected, the level of surface CD81 mAb decreased faster after TPA treatment (Fig. 3B). However, in both cases, the total level of cell-associated CD81 mAb remained very similar. This result, together with the confocal microscopy analysis, indicates that TPA accelerates CD81 endocytosis. Thus ~50% of CD81 was internalized after 1 h TPA treatment as compared to only ~20% in non-treated cells.

CD81 could be easily detected by Western-blot during the first 6 h to TPA treatment. (Fig. 4A). However, after 24 h treatment, there was a marked reduction of CD81 in the cells. At these latter time points, the down-regulation of CD81 is due to an inhibition of synthesis as determined by a pulse chase experiment (Fig. 4B). As a control the synthesis of CD9 was strongly increased in the same cells. Quantitative RT-PCR experiments demonstrated a two-fold reduction of CD81 mRNA level while a large increase of the level of CD9 mRNA was observed, consistent with our previous study [Le Naour et al., 1997] (data not shown).

Release of Tetraspanins From K562 cells Via Exosomes and Upregulation After TPA Treatment

In preliminary studies we observed that a fraction of CD81 was present in the supernatant of K562 cells (data not shown). The MW of this CD81 fraction was identical to that of cell membrane-bound CD81 excluding the possibility of a proteolytic cleavage. We thus examined whether this fraction of CD81 could be present on exosomes produced by K562 cells. Exosomes can be purified by sequential centrifugations [Raposo et al., 1996]. This approach yields five pellets with P1 corresponding to the cells and P5 being highly enriched in exosomes. Examination of the P5 pellet by electron microscopy showed that this fraction indeed contained small 50–100 nm vesicles resembling exosomes (Fig. 5B). Analysis of the protein composition of the P5 fraction by western-blot further confirmed that these vesicles are exosomes (Fig. 5A). This fraction is indeed enriched in

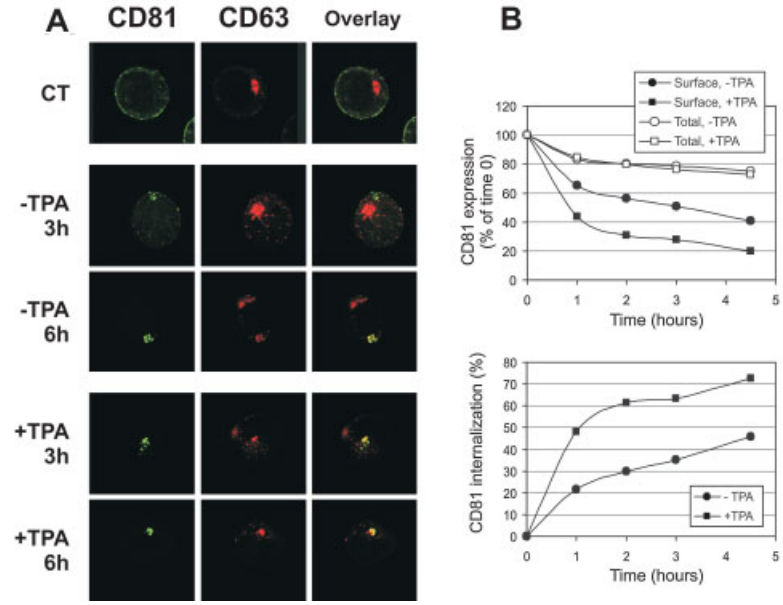

Fig. 3. TPA accelerates endocytosis of CD81. **A**: Internalization of CD81. K562 cells were incubated with the CD81 mAb TS81 at 4°C and incubated at 37°C for the indicated time in the presence or not of TPA. They were then attached to polylysine-coated coverslips, fixed with acetone and labeled with the CD63 mAb TS63. The mAb TS81 and TS63 were revealed by a combination of alexa-488-labeled anti-mouse IgG2a (TS81, green) and alexa-568-labeled anti-mouse IgG1 (TS63, red) before confocal fluorescence microscopy analysis. Bar 10 μm. **B**: TPA accelerates CD81 internalization. K562 cells were incubated at 4°C in the presence of Alexa 488-labeled TS81 mAb, washed and incubated at 37°C for the indicated time in the presence or absence of TPA. They were then stained using a phycoerythrin-labeled goat anti-mouse polyclonal antibody, and analyzed by flow-cytometry. In this experiment, the Alexa 488 fluorescence provides a relative measure of total cell-associated TS81 mAb (**top panel**, ''total,'' and the phycoerythrin fluorescence a relative measure of TS81 still bound to the cell surface (top panel, ''surface''). In the **lower panel**, CD81 internalization was quantified as described in Material in Method section.

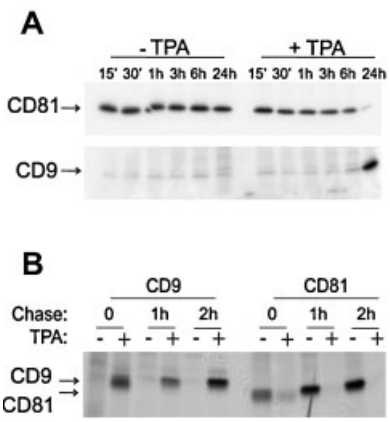

Fig. 4. Reduced synthesis of CD81 following TPA treatment. **A**: The amount of CD81 or CD9 present in cells treated or not with TPA for the indicated time was analyzed by Western-blot. **B**: Pulse-chase analysis of CD9 and CD81 synthesis K562 cells treated or not with TPA for 20 h were metabolically labeled with ^{35}S-methionine and ^{35}S-cysteine for 15 min. The cells were then washed and chased for the indicated time, before immunoprecipitation of CD9 or CD81. The results show a strong induction of CD9 synthesis and a strong reduction of CD81 synthesis after TPA treatment.

TfR, a classical marker of exosomes, previously reported to be present on exosomes produced by K562 cells [Savina et al., 2002], and in annexin II, previously identified in exosomes produced by dendritic cells [Thery et al., 1999]. It also contains the ESCRT protein Tsg101 as well as several tetraspanins such as CD53, CD63, and CD81. We did not detect the heat shock protein Hsc-70 or the GPI-anchored molecule CD55 in the exosome fraction, contrasting with previous studies. Hsc-70 was found regularly in exosomes produced by various cells, including K562 cells [Savina et al., 2002; Wubbolts et al., 2003], while CD55 was observed on exosomes produced by erythrocytes [Rabesandratana et al., 1998]. Our data do not exclude the possibility that a small fraction of these molecules is present on exosomes produced by K562 cells, but indicate that they are not enriched in this fraction, in contrast with the TfR.

While CD81, CD9P-1, and TfR were all diminished at cell surface after TPA treatment, they were subjected to different fates. TPA treatment induced an increase of the amount of

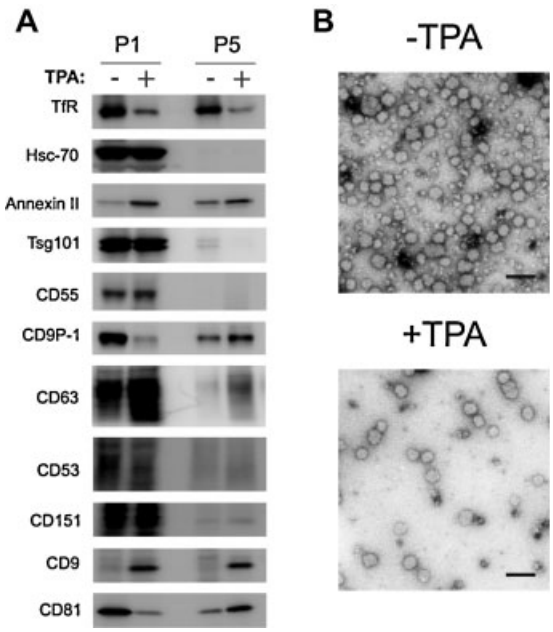

Fig. 5. K562 cells produce exosomes enriched in several tetraspanins and CD9P-1. **A**: The supernatants of K562 cells treated or not with TPA for 20 h were subjected to differential centrifugation. The last pellet (P5), enriched in exosomes, was analyzed by Western-blot in comparison with the cell extract (P1). **B**: Exosomes from control or K562 cells treated with TPA for 20 h were observed by electron microscopy. Bar 100 nm.

CD81 and CD9P-1 in the P5 fraction, but a decrease of TfR. This diminution of TfR in the exosome fraction (an approximately fourfold diminution) is of the same magnitude as the diminution of material in this fraction as determined using the Bradford assay (data not shown) and direct examination by electron microscopy (Fig. 5B). It is interesting to note that the large increase of CD9 expression at cell surface following TPA treatment was associated with a large increase of the localization in exosomes.

The TPA-Induced Targeting of CD81 and CD9P-1 to Exosomes Does Not Require a Neo-Synthesized Pool

To characterize the origin of CD81 and CD9P-1 molecules present in exosomes, the cells were treated by Brefeldin A (BFA), a drug that prevents the egress from the ER to the Golgi [Jackson, 2004]. This drug did not have significant effect on the steady state levels of TfR, CD9P-1, CD81, and CD9 (Fig. 6). It resulted in the apparition of a second band revealed by the anti CD9P-1 mAb, which probably corresponds to a molecule lacking extensive glycosylation. BFA treatment had little effect on the localization of CD81 in exosomes produced by non-treated K562 cells but diminished the amount of TfR and CD9P-1 that could be recovered in exosomes. This differential effect could reflect the half-life of the proteins or the effect of BFA on the endosomal compartment [Lippincott-Schwartz et al., 1991]. Importantly, BFA treatment blocked the increase of CD9 in the exosome fraction observed after TPA treatment, but not that of CD9P-1 and CD81. This shows that the increased amount of CD81 or CD9P-1 in exosomes after TPA treatment is not dependent on a neosynthesized pool. The effect of BFA on CD9 is consistent with the strong increase of CD9 synthesis upon TPA treatment (Figs. 1 and 4). The decrease of CD9 Mr upon BFA treatment is most likely due to the inhibition of palmitoylation [Charrin et al., 2002; Yang et al., 2002]. The effect of cycloheximide pretreatment was markedly different since this translation inhibitor inhibited the TPA-induced enrichment of CD81 and CD9P-1 into exosomes. The differential effects of BFA and cycloheximide on the enrichment of these two molecules in exosomes strongly suggest that protein synthesis of CD81 and CD9P-1 is not necessary, but that this enrichment is regulated by a protein whose synthesis is required during the time of treatment.

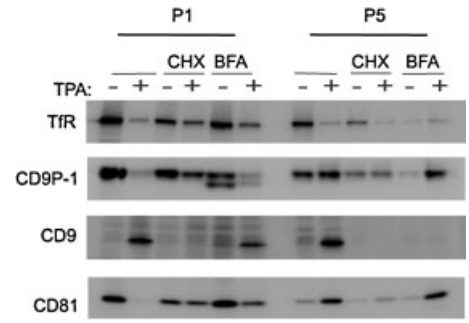

Fig. 6. Effect of cycloheximide and Brefeldin A on the presence of various proteins in exosomes. K562 cells were treated or not with TPA for 20 h. Then, the supernatants were subjected to differential centrifugation and the last pellet (P5) was analyzed by Western-blot in comparison with the cell extract. In some samples cycloheximide (CHX, 100 ng/ml) or Brefeldin A (BFA, 10 μg/ml) were added 30 min before addition of TPA.

Pattern of Surface Proteins Associated With CD81 in K562 Cells and Association of CD81 With the TfR

As a first step to test the hypothesis that tetraspanins could allow the sorting of the molecules to which they associate into exosomes,

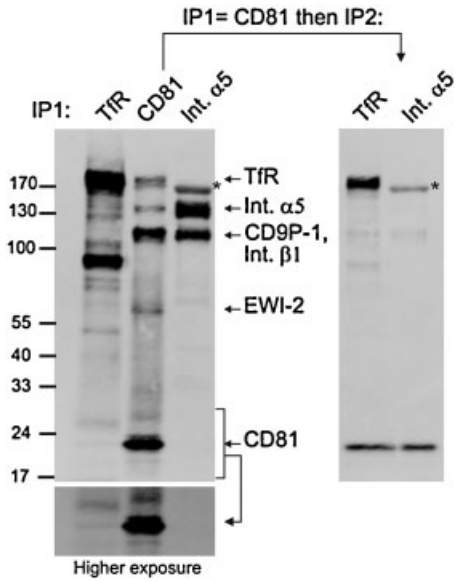

Fig. 7. Association of a fraction of TfR with CD81. K562 cells were biotin-labeled, lysed in Brij 97, and immunoprecipitations (IP1) were performed with mAb directed to the TfR, CD81, and the integrin α5 as indicated. A higher exposure of the bottom of the blot is shown. In the **right panel** (IP2), the proteins co-immunoprecipitated with CD81 were eluted with 1% Triton X-100 and subjected to a second immunoprecipitation with the anti-TfR and anti integrin α5 mAb. The additional band of ~95 kDa present in the anti-TfR immunoprecipitate but not identified in the figure corresponds to a TfR monomer as it is labeled by a TfR mAb (data not shown). The * indicates an Ig band. CD9P-1 in the CD81 immunoprecipitate and the integrin β1 subunit in the integrin α5 immunoprecipitate co-migrate.

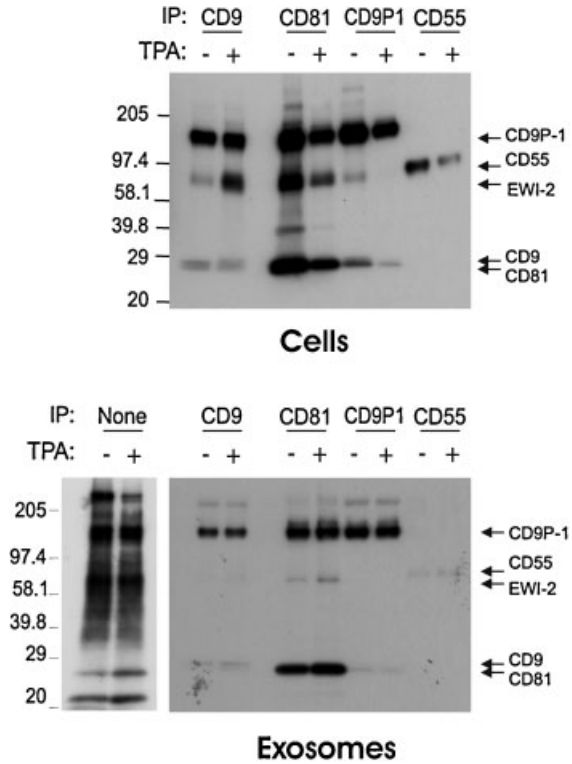

Fig. 8. Association of CD81 with CD9P-1 in exosomes. K562 cells were Biotin-labeled before treatment or not with TPA for 20 h. Then, the cells (**top**) and exosomes (**bottom**) were lysed in Brij 97 and immunoprecipitations with CD9, CD81, anti-CD9P-1 or CD55 mAbs were performed.

we characterized the pattern of molecules associated with CD81 in K562 cells. As shown in Figure 7, the two main molecules co-immunoprecipitated with CD81 after lysis with Brij 97 (a mild detergent widely used to study the interaction of membrane proteins) had a Mr of 125 and 63 kDa (non-reducing conditions) and corresponded to CD9P-1 and EWI-2, respectively. A ~180 kDa band present in the CD81 immunoprecipitate co-migrated with the disulfide-bound TfR homodimer. Reciprocally, a band co-migrating with CD81 was present in the TfR immunoprecipitate but not the integrin α5 immunoprecipitate. To check that the ~180 kDa band present in the CD81 immunoprecipitate is indeed the TfR, the proteins co-immunoprecipitated with CD81 were eluted in Triton X-100 and subjected to a second immunoprecipitation. We could immunoprecipitate the TfR but not the integrin α5 from the eluted material (Fig. 7, right panel) indicating an interaction of CD81 with a fraction of this receptor. Similar results where obtained after cell lysis with Brij 58.

CD81 and CD9P-1 Present on Exosomes Originate From the Cell Surface and Remain Associated

To directly demonstrate that a fraction of CD81 and CD9P-1 present at cell surface at the time of treatment was targeted to exosomes, K562 cells were surface-labeled with biotin. Then, after TPA treatment or not, immunoprecipitations were performed on cell (Fig. 8, top) and exosome (Fig. 8, bottom) extracts obtained after lysis with Brij 97 (Fig. 8). The presence of these molecules in the immunoprecipitates was determined by blotting with streptavidin peroxydase. Thus only the fraction present at cell surface at the time of labeling is analyzed.

Both biotin-labeled CD81 and CD9P-1 were clearly detected in exosomes (Fig. 8, bottom). Only a faint band of CD9 could be observed. After TPA treatment, the band corresponding to CD81 was of higher intensity, indicating a higher recruitment of CD81 from the plasma membrane. In contrast, the intensity of the band corresponding to CD9 was not changed

after TPA treatment, contrasting with the sharp increase of CD9 in the exosome fraction after TPA treatment as determined by Western-blotting (Fig. 5). Thus, most of the CD9 molecules present in exosomes after TPA treatment were not at cell surface when TPA was added to the cells. This is consistent with the observation that BFA blocks the increase of CD9 in exosomes (Fig. 6). Analysis of the entire pattern of biotinylated proteins in exosomes (Fig. 8, bottom, left) showed that TPA treatment did not dramatically change the pattern of major (biotinylated) proteins present in exosomes showing the specificity of CD81 enrichment in this fraction. Note that other tetraspanins are poorly labeled with biotin and therefore are not visualized.

As shown in Figure 8, CD81 could strongly precipitate CD9P-1 not only from the cell lysate (Fig. 8, top) but also from exosome lysate (Fig. 8, bottom). CD81 immunoprecipitated as much CD9P-1 as did the anti-CD9P-1 mAb indicating that in these cells the bulk of CD9P-1 molecules is associated with CD81. CD9P-1 only co-immunoprecipitated a fraction of CD81 molecules, from both cell and exosomes extracts. This is consistent with the expression level of CD9P-1 being lower than that of CD81 in K562 cells (data not shown). In contrast, the co-immunoprecipitation of EWI-2 with CD81 was strongly reduced in exosomes. It is noteworthy that although the amount of CD9 in the exosome fraction was strongly increased after TPA treatment, the CD9 mAb did not co-immunoprecipitate higher levels of biotin-labeled CD81 and CD9P-1. This result indicates that the CD9 pool synthesized after TPA addition and

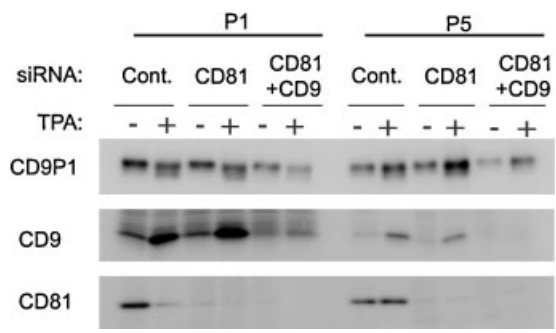

Fig. 9. CD9P-1 is sorted into exosomes in the absence of CD9 and CD81. K562 cells stably expressing CD9P-1 were transfected with siRNA directed to CD81, CD9 or a control siRNA, as indicated. The cells were then treated or not with TPA for 20 h and the presence of CD9P-1 and CD81 in exosomes was analyzed by Western-blot.

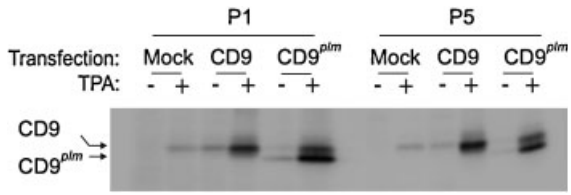

Fig. 10. Palmitoylation of CD9 is not required for sorting into exosomes. K562 cells were transfected with a vector encoding CD9 or a mutant CD9 lacking all palmitoylation sites ($CD9^{plm}$). The Mr of this CD9 mutant is slightly lower than that of WT CD9. The cells were treated or not with TPA for 20 h and the presence of CD9 and $CD9^{plm}$ in exosomes was analyzed by Western-blot.

targeted to exosomes does not associate with the pre-existing CD81/CD9P-1 complex, and clearly demonstrates that the observed associations do not result from a post-lysis artefact.

CD9P-1 is Sorted to Exosomes in the Absence of CD81 and CD9

The above data showed that CD81 and CD9P-1 in exosomes come from a surface pool and specifically remain associated. This made possible the hypothesis that CD81 could allow the targeting of CD9P-1 into exosomes. K562 cells overexpressing CD9P-1 were treated with specific siRNA to silence CD81 alone or along with CD9. Both tetraspanins were silenced because CD9P-1 is a molecular partner of both CD9 and CD81 [Charrin et al., 2001; Stipp et al., 2001b], The knock-down of these molecules was checked by flow-cytometry (data not shown) and Western-blot (Fig. 9). CD9P-1 was still recovered in the exosome fraction after silencing of CD9 and CD81 while as a control CD9 or CD81 could no longer be detected in this fraction. Thus the sorting of CD9P-1 into exosomes is independent from these two tetraspanins.

Palmitoylation of CD9 Is Not Required for Exosome Targeting

The demonstration that the lipid composition of B-cell derived exosomes resembles that of rafts [Wubbolts et al., 2003], with in particular enrichment in cholesterol, led to the suggestion that some proteins may be sorted into the inwardly budding vesicles of MVB through a particular interaction with lipids [De Gassart et al., 2003; Wubbolts et al., 2003]. Because tetraspanins interact with cholesterol, and a non-palmitoylated mutant CD9 was defective in the interaction with this lipid [Charrin et al., 2003c], we examined a possible role of

palmitoylation in sorting CD9 into exosomes (Fig. 10). Both WT and non-palmitoylated CD9 ($CD9^{plm}$) were stably expressed in K562 cells. The non-palmitoylated CD9 molecule migrates slightly faster than WT CD9 [Charrin et al., 2002]. Both proteins were sorted to exosomes to the same extent, in the presence or not of TPA. The increase of the ectopically expressed molecule after TPA treatment is likely to be due to an activation of the CMV promoter in the plasmid [Chan et al., 1996].

DISCUSSION

In the course of reticulocyte maturation, elimination of the TfR and other surface proteins relies on internalization and targeting to MVB, which release internal vesicles after fusion with the plasma membrane [Johnstone, 2005]. Whether other integral proteins present on exosomes also originate from the plasma membrane and whether they are targeted through a mechanism similar to that of TfR is not known. This question is especially pertinent for tetraspanins which not only accumulate in the plasma membrane at the steady-state level, but also, for some of them, especially CD63, in intracellular compartments such as lysosomes and MVB. The generation of exosomes, and therefore the targeting of the proteins they contain is probably a continuous process. We reasoned that the availability of a model where the targeting of molecules into exosomes could be induced would facilitate the study of the mechanisms underlying targeting into exosomes.

Activation of PKC by TPA induced the rapid internalization of CD81 in K562 cells. TPA treatment of K562 cells was shown to promote the interaction of tetraspanins (including CD81) with PKCα and PKCβ2 in K562 cells while activation of Jurkat T cells with anti-CD3 antibodies was shown to induce the association of PKCα with tetraspanins [Zhang et al., 2001]. Because no PKC binding site could be identified in tetraspanins [Zhang et al., 2001], we could not directly address the possible contribution of this interaction in the TPA-induced internalization of CD81. It is however unlikely that the TPA-induced internalization of CD81 is a consequence of this interaction as TPA causes the rapid internalization of various surface molecules ([Klausner et al., 1984; DiSanto et al., 1989; Moraru et al., 1990; Dietrich et al., 1994] and this article). In this regard, the TfR is rapidly internalized following PKC activation (including in K562 cells) [Klausner et al., 1984] even when the PKC phosphorylation site in TfR is mutated [Zerial et al., 1987]. Rather, PKC is thought to activate the internalization machinery. This is consistent with TPA augmenting pinocytosis in K562 cells [Schonhorn et al., 1995].

A unique effect of TPA on TfR, CD81, and CD9P-1 was that the expression levels of these three molecules were lower after 24 h treatment than after 3 h. Exposure of K562 cells to TPA was indeed shown to reduce synthesis of TfR [Schonhorn et al., 1995] and we have now determined that it induces a down-regulation of CD81 protein synthesis, in relation with a diminution of CD81 mRNA levels, as determined by quantitative RT-PCR. This two-step down-regulation of CD81 (internalization and reduced synthesis) is very similar to what has been reported in lymphoid T cells upon activation by CD3 and CD28 mAb [Fritzsching et al., 2002]. Interestingly, like TPA treatment, activation of T cells causes an activation of serine/threonine kinases of the PKC family [Bauer and Baier, 2002]. The synchronization of internalization and the strong reduction of synthesis make the K562 model especially useful to study the fate of CD81 molecules after internalization.

TPA increased the localization of several tetraspanins into exosomes. For some molecules such as CD9 and CD82 (data not shown), this is due to an increased synthesis. Indeed, the TPA-induced increase in CD9 release was blocked by BFA, and very little CD9 present in exosomes was at cell surface at the time of treatment. In contrast, TPA reduced the expression of CD81, CD9P-1, and TfR, but had strikingly different effects on their targeting into exosomes. This treatment increased the release into the extracellular medium of CD9P-1 and CD81, but decreased the amount of TfR recovered in this fraction. The bulk of CD81 and CD9P-1 molecules present on exosomes after TPA treatment comes from a pre-existing pool, as treatment of cells with BFA did not diminish the release of these molecules in the supernatant. Moreover, at least a fraction of these molecules was present at the cell surface at the time when TPA was added. Indeed, when surface proteins were biotin-labeled before TPA treatment, a fraction of labeled-CD81, CD9P-1 (and other molecules) was recovered in the exosome fraction. These data show that sorting of TfR and

CD81 (and CD9P-1) into exosomes is regulated by different mechanisms.

How tetraspanins are targeted to exosomes is so far completely unknown. In contrast to other tetraspanins such as CD63 and CD82, CD81 and CD9 do not have a tyrosine-based YxxΦ motif. These molecules interact with cholesterol [Charrin et al., 2003c] and GM3 [Ono et al., 2001], and can be present in a detergent-resistant membrane environment different from rafts [Claas et al., 2001; Charrin et al., 2002]. On the other hand, the lipid composition of B-cell derived exosomes resembles that of rafts [Wubbolts et al., 2003], displaying a particular enrichment in cholesterol and GM3. This led to the suggestion that some proteins may be sorted into the inwardly budding vesicles of MVB through a particular interaction with lipids [Wubbolts et al., 2003; De Gassart et al., 2004]. Protein palmitoylation is a way to increase the interaction of proteins with lipids [Resh, 1999], and tetraspanins are highly palmitoylated [Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002]. This post-translational modification contributes to the interaction of tetraspanins with each other and possibly to the interaction with cholesterol [Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002]. However, palmitoylation does not play a major role in CD9 sorting into exosomes since a CD9 mutant lacking all palmitoylation sites was sorted into exosomes.

Many tetraspanin-associated proteins are present in exosomes, such as a subset of integrins [Wubbolts et al., 2003], ADAM-10 [Le Naour et al., 2006; Stoeck et al., 2006], MHC molecules (for review see [Stoorvogel et al., 2002; Thery et al., 2002b; De Gassart et al., 2004]) and CD9P-1 (our study). This led to the suggestion that tetraspanins may help in sorting into exosomes some of the proteins to which they interact with [Stoorvogel et al., 2002; De Gassart et al., 2004]. In this regard, the intracellular traffic of some tetraspanin-associated proteins can be regulated by the tetraspanins to which they associate (for review, see [Berditchevski and Odintsova, 2007]). We have demonstrated in this study that an important fraction of CD9P-1, a common molecular partner of CD9 and CD81, still traffics to exosomes after knocking-down these two tetraspanins. Therefore, the role of CD81 is not to target CD9P-1 to exosomes. It is unlikely that CD9P-1 could in turn allow the sorting of CD9 or CD81 into exosomes as these tetraspanins can be found in exosomes produced by cells not expressing this molecule such as lymphoid cells.

CD81 plays a major role in the infection of hepatocytic cells by two major infectious agents, the hepatitis C virus and *Plasmodium*, the parasite causing Malaria. Recently the demonstration that a CD81 mAb could inhibit model pseudoparticles entry after attachment to the cells led to the suggestion that CD81 functions as a post-attachment entry co-receptor [Cormier et al., 2004]. However, previous studies have shown that the internalization rate of CD81 is slow [Petracca et al., 2000], and comparable to the internalization of CD81 in K562 cells. The finding that this internalization can be accelerated upon proper stimulation may have implications for better understanding the role of CD81 during HCV infection.

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