

# PLD2 is enriched on exosomes and its activity is correlated to the release of exosomes

Karine Laulagnier<sup>a</sup>, David Grand<sup>a</sup>, Arnaud Dujardin<sup>a</sup>, Safouane Hamdi<sup>a</sup>,  
Hélène Vincent-Schneider<sup>b</sup>, Danielle Lankar<sup>b</sup>, Jean-Pierre Salles<sup>a</sup>, Christian Bonnerot<sup>b</sup>,  
Bertrand Perret<sup>a</sup>, Michel Record<sup>a,\*</sup>

<sup>a</sup>Département Lipoprotéines et Médiateurs Lipidiques, INSERM U563, CPTP, Bât C, CHU Purpan, Place Baylac, BP3028, 31024 Toulouse Cedex 3, France

<sup>b</sup>INSERM U520, "Biologie Cellulaire de l'Immunité anti-tumorale", Institut Curie, 26 rue d'Ulm, 75248 Paris Cedex 05, France

Received 28 June 2004; accepted 28 June 2004

Available online 14 July 2004

Edited by Sandro Sonnino

**Abstract** Exosomes are small vesicles secreted by different immune cells and which display anti-tumoral properties. Stimulation of RBL-2H3 cells with ionomycin triggered phospholipase D2 (PLD2) translocation from plasma membrane to intracellular compartments and the release of exosomes. Although exosomes carry the two isoforms of PLD, PLD2 was enriched and specifically sorted on exosomes when overexpressed in cells. PLD activity present on exosomes was clearly increased following PLD2 overexpression. PLD2 activity in cells was correlated to the amount of exosome released, as measured by FACS. Therefore, the present work indicates that exosomes can vehicle signaling enzymes.

© 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

## 1. Introduction

Phospholipase D (PLD) hydrolyzes phosphatidylcholine to generate choline and phosphatidic acid (PA). PA is an important lipid second messenger implicated in many cellular functions [1], including exocytosis, endocytosis, remodeling of the actin cytoskeleton, and which also directly activates a range of target proteins such as mTOR [2]. Since PA is a fusogenic lipid [3], it has been implicated in different steps of vesicular trafficking and intracellular membrane fusion events [4]. For example, PLD is involved in secretion mechanisms in granulocytes [5] and RBL mast cells [6]. Also, inhibitors of serine/threonine kinases that regulate PLD activity suppress both PLD activity and secretion in these cells [7]. Two main isoforms of PLD, PLD1 and PLD2, have been characterized, which are differently regulated but are both dependent on phosphatidylinositol-4,5-bisphosphate [1]. PLD1 is activated by GTPases of ARF family such as Arf1 and of Rho families (RhoA, cdc42 and Rac), and by protein kinases C. Conversely,

PLD2 is constitutively active and weakly sensitive to Arf1 [1]. Synucleins [8] and actin [9] are known as cellular inhibitors of PLD2 activity but PLD2 regulation is still unclear. Each isoform seems to be differently localized in cells. PLD1 is present on intracellular compartments corresponding to granules or Golgi apparatus [10], whereas PLD2 has been observed preferentially on plasma membrane [6]. Due to its low abundance, the intracellular localization of PLD2 has been studied only indirectly through overexpression of chimeric proteins like GFP-tagged PLD2 [6]. Nevertheless, it has also been shown that a significant fraction of endogenous PLD2 localized to the perinuclear Golgi region and was also distributed throughout dense cytoplasmic puncta [11].

We have investigated the role of PLD2 in intracellular vesicular trafficking in mast cells and more precisely in the secretion of immuno-active vesicles, exosomes. Exosomes are small vesicles (60–90 nm) formed in multivesicular bodies (MVB) and secreted when MVB fuse with plasma membrane [12]. These vesicles have attracted much interest, since Zitvogel et al. [13] have shown that dendritic cells-derived exosomes endowed immune anti-tumoral activities in mice. Clinical trials are currently in progress.

In the present work, we have observed the enrichment of an active PLD2 on exosomes secreted by RBL-2H3 cells and shown that PLD2 was necessary to obtain maximal exosome secretion.

## 2. Materials and methods

### 2.1. Chemical reagents and antibodies

RPMI 1640, DMEM, PBS, penicillin, streptomycin and L-glutamine were purchased from Biowhittaker. FCS was from Gibco (Paisley, UK). All solvents, alcohols were from Merck Eurolab (VWR). 4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY)-PC and BODIPY-ceramide (Molecular Probes) were stored in ethanol at –20 °C. All chemical reagents were from Sigma (St. Louis) except glycine (Eurobio). Rabbit polyclonal anti-PLD antibody (N-PLD4) was from Johnson Pharmaceutical Research Institute (Raritan, NJ, USA) and was kindly supplied by Dr. D. Uhlinger. This antibody was raised against the sequence 523–537 of human PLD2, which was found 100% identical with rat PLD2 and 75% identical with rat PLD1. The HA.11 monoclonal mouse anti-HA antibody (clone 16B12) was from BabCo (Eurogentec). Secondary antibodies labeled with horseradish peroxidase (HRP, goat anti-rabbit) or with rhodamine (goat anti-mouse)

\* Corresponding author.

E-mail address: record@cict.fr (M. Record).

**Abbreviations:** BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; NBD, 2-6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino; MVB, multivesicular bodies

were from Chemicon and Beckman-Coulter, respectively. Methyl arachidonoyl fluorophosphonate (MAFP) was from Calbiochem.

### 2.2. Cells

RBL-2H3 cells were grown as previously reported [14]. Cells were kept either adherent, or in suspension under constant agitation in spinner bottles (VWR). RBL cells overexpressing active (RBL PLD2+) or inactive (RBL PLD2-) PLD2 were obtained by electroporation (250 V, 500  $\mu$ F) of cells with linearized pcDNA3.1 vector containing the HA-tagged cDNA of murine PLD2 with a catalytic active or inactive (K758R) sequence. PLD2 overexpressing cells were selected with geneticin (500  $\mu$ g/ml) added in regular cultures, but suppressed in spinner bottle cultures for exosome preparation.

### 2.3. Exosomes preparation

Wild type RBL cells, RBL PLD2+ or RBL PLD2-, were grown in suspension and degranulation was triggered with ionomycin (final concentration 1  $\mu$ M) during 20 min at 37 °C. Exosomes were then recovered and purified by differential centrifugations from supernatants recovered after stimulation as described [14,15]. The final pellet referred to as exosomes was taken up in PBS for further analyses. Usually, 300  $\mu$ g of exosomal proteins [14] was obtained from  $1 \times 10^9$  RBL Wt cells. Quality of preparations was routinely checked by electron microscopy (D. Lankar, Institut Curie [15]).

### 2.4. Cytofluorimetry analysis of *in vitro*-labeled exosomes

Exosomes were labeled with 4.8  $\mu$ M BODIPY-ceramide, BODIPY-PC or 2-6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino (NBD)-PC in PBS for 30 min at 37 °C in the dark. Excess of fluorescent lipids was removed by two successive ultracentrifugations at 110 000  $\times$ g for 70 min at 4 °C in a TLX100 ultracentrifuge (Beckman Coulter). Pelleted fluorescent exosomes were taken up in PBS and fluorescence was measured by spectrofluorimetry ( $\lambda_{exc} = 475$  nm;  $\lambda_{em} = 515$  nm).

For FACS analyses, pelleted labeled exosomes were incubated at room temperature 15 min with 10  $\mu$ l of aldehyde-sulfate-coated latex beads (Interfacial, Dynamics Corp., Portland, USA) and then for 1 h in 1 ml of PBS [15]. Free aldehyde-sulfate groups were then saturated by 100 mM of glycine for 30 min at room temperature [15]. Beads carrying fluorescent exosomes were then washed twice in FACS buffer (0.01%  $\text{NaN}_2$ , 1% FCS in PBS) by two centrifugations for 3 min at 2000 rpm and finally analyzed by FACS with the settings used for FITC. Fluorescence was proportional to known amounts of exosomes up to 30  $\mu$ g proteins.

### 2.5. Measurement of PLD activity by HPLC

Production of phosphatidylethanol (transphosphatidylolation reaction) [1] was monitored by HPLC using fluorescent phosphatidylcholine (BODIPY-PC) as PLD substrate.  $1 \times 10^6$  sonicated RBL cells or 100  $\mu$ g exosomes were incubated, respectively, 30 and 60 min with 1.1  $\mu$ M of BODIPY-PC and 1% ethanol in 1 ml PBS with 2 mM  $\text{Ca}^{++}$ /Mg $^{++}$ , at 37 °C with 10  $\mu$ M MAFP (a phospholipase A2 inhibitor) and 10  $\mu$ l protease inhibitor cocktail (P8340, Sigma). Then, fluorescent lipids were extracted with 1 ml butanol-1. Separation of fluorescent BODIPY-PC-derived products was realized according to the procedure described by Kemken et al. [16].

### 2.6. Western blotting analyses

Cells and exosomes were lysed in sample buffer (80 mM Tris-HCl, glycerol 10%, SDS 10%,  $\beta$ -mercaptoethanol 2%, w/v; bromophenol blue 5%, v/v) and sonicated. 40  $\mu$ g of proteins was then separated on a 6.5% SDS-polyacrylamide gel and transferred on to a nitrocellulose membrane. Membranes were saturated with 10% non-fat milk in PBS-0.1% Tween 20 for 1 h at room temperature and blotted with rabbit or mouse primary antibodies against PLD or HA, respectively, in 1% non-fat milk in PBS-0.1% Tween 20 at room temperature for 45 min. Membranes were then washed and incubated in the same buffer with HRP-labeled, anti-rabbit IgG or anti-mouse IgG secondary antibodies. The signal was detected by enhanced chemiluminescence system.

### 2.7. Immunofluorescence microscopy experiments

Resting RBL PLD2+ cells were grown overnight on glass/Teflon coverslips (VWR) in complete medium ( $1 \times 10^4$  cells/50  $\mu$ l). Cells were then fixed 20 min with 5% PBS/paraformaldehyde (w/v) at room temperature, and permeabilized 15 min at room temperature with 0.05% saponin (w/v) in RPMI, 10% BSA (w/v), and 10 mM glycine.

Cells were then labeled at room temperature with anti-HA antibody in PBS for 45 min and washed before incubation with rhodamine-labeled anti-mouse secondary antibody in PBS for 45 min in the dark. For labeling of activated cells, cells were plated, stimulated with ionomycin (15 min, 37 °C), and then treated as for resting cells. Samples were analyzed with a confocal laser scanning microscope (Zeiss LSM510).

## 3. Results

We analyzed PLD isoforms expression on exosomes comparatively to parent cells. As shown in Fig. 1A, PLD1 is more expressed than PLD2 in RBL, whereas on exosomes the two isoforms are equally expressed, indicating that PLD2 is enriched on exosomes compared to total parent cells. We then overexpressed an HA-tagged PLD2 in RBL and also observed

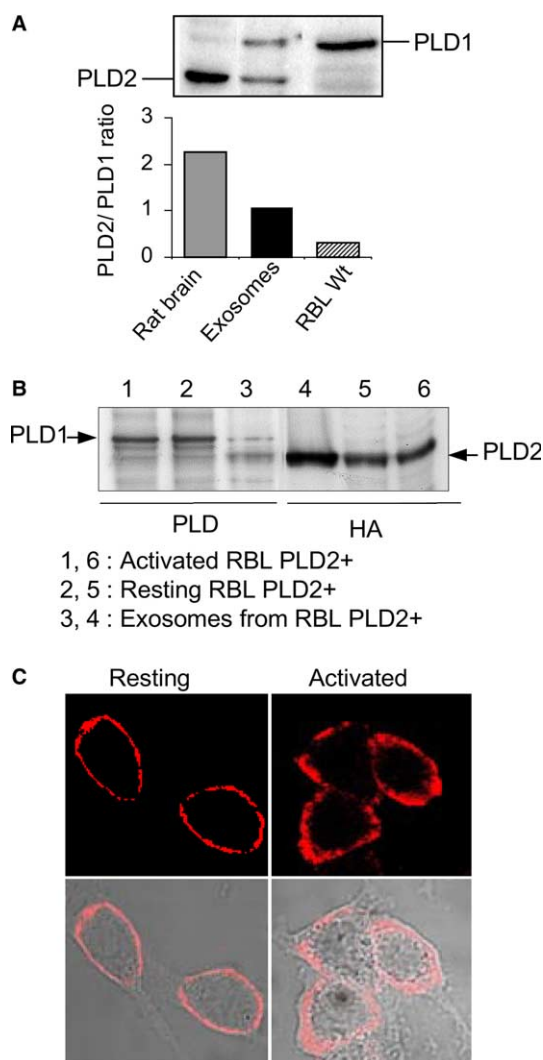


Fig. 1. (A) Exosomes and RBL-2H3 cells were analyzed by Western blotting with polyclonal NPLD4 anti-PLD antibody. Rat brain was used as PLD2 control. (B) Resting and ionomycin-activated RBL cells overexpressing HA-tagged PLD2 (RBL PLD2+) and exosomes from RBL PLD2+ were analyzed by Western blotting using NPLD4 or anti-HA antibodies. (C) HA-tagged PLD2 localization was analyzed by immuno-fluorescence microscopy in resting or ionomycin-activated RBL PLD2+ cells. Lower panels in (C) are merge of transmission and fluorescence.

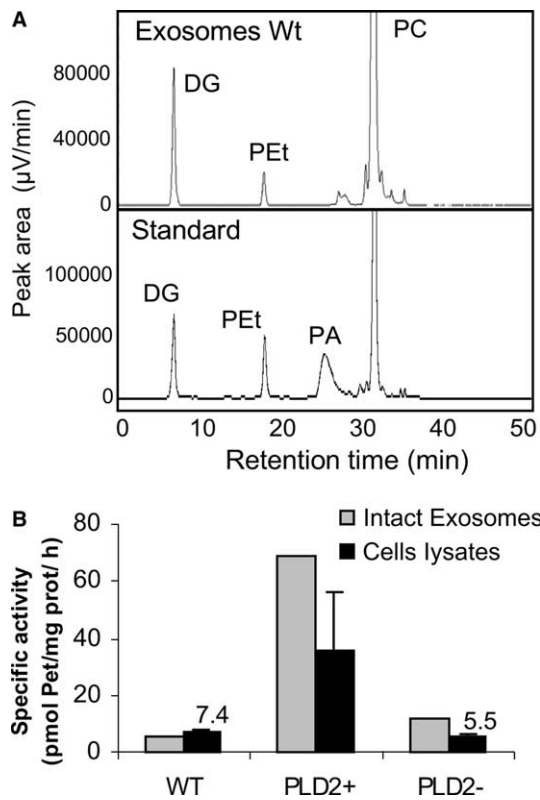


Fig. 2. (A) Typical chromatograms representatives of fluorescence HPLC measurement of PLD activity. (B) Quantification of PLD specific activity on exosomes from WT, PLD2+, PLD2- RBL cells and corresponding parent cells. Results are means of 2–3 experiments  $\pm$  S.E.M.

an enrichment of this protein in exosomes as compared to cells (Fig. 1B). We next tracked HA-tagged PLD2 in cells by fluorescence microscopy. In resting RBL, HA-PLD2 was located on the plasma membrane (Fig. 1C, left panels), as already reported [6]. When degranulation was triggered, we noticed a redistribution of PLD2 from the plasma membrane towards the cell interior (Fig. 1C, right panels), which might direct PLD2 on exosomes.

Exosomes recovered from stimulated RBL Wt contained an active PLD (Fig. 2) as evidenced by the presence of BODIPY-

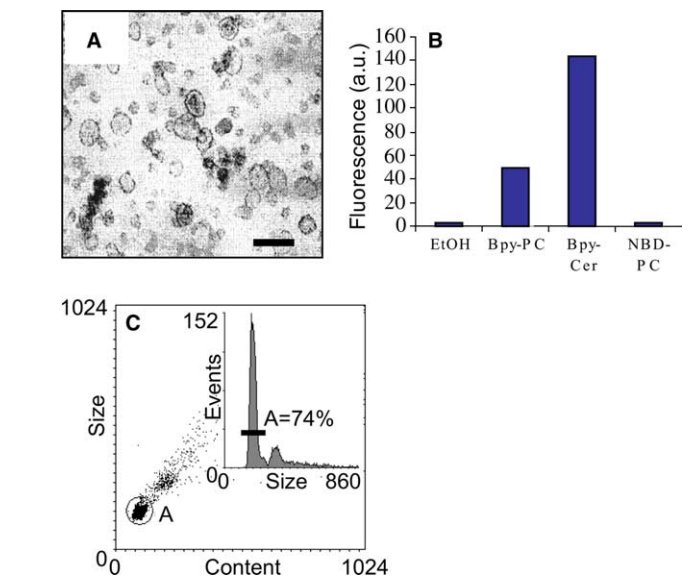
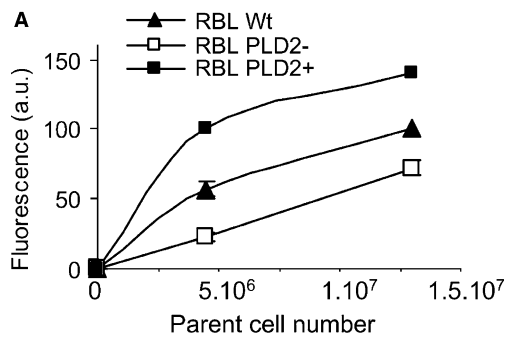


Fig. 3. (A) Electron microscopy analysis of exosomes from RBL Wt cells. Bar = 100 nm. (B) Exosomes were labeled with BODIPY-PC, BODIPY-ceramide or NBD-PC and fluorescence was monitored by spectrofluorimetry (a.u., arbitrary units). (C) BODIPY-ceramide-labeled exosomes were loaded on latex beads and analyzed by FACS. Dot plots showing forward scatter (size) vs. side scatter (content) are presented. Inset: Exosomes bound on monomeric beads (circled in (A)) were quantified.

PEt (Fig. 2A). DG but not PA was noticed on exosomes, suggesting the presence of a PA-phosphatase. The global PLD specific activity (involving both PLD1 and 2) in control Wt samples was similar in exosomes and parent cells (Fig. 2B). As compared to Wt samples, overexpression of PLD2 led to a specific PLD activity about 10 and five times higher in exosomes and cells, respectively. Overexpression of inactive PLD2 led to about 20% decrease in PLD specific activity in cells (7.4 vs. 5.5 pmol/mg/h), but no clear effect on exosome PLD activity was noticed.

We next investigated whether these modifications of PLD2 expression and PLD activity in RBL cells could influence degranulation. For this purpose, we improved a previously described method to measure the amount of exosomes released [15]. Quality of exosomes preparation was checked by electron microscopy. A typical preparation, consistent with previous

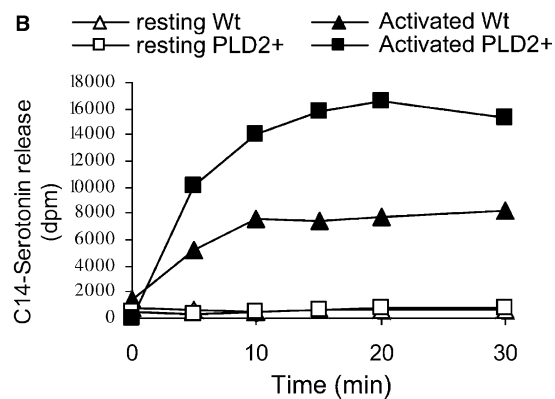


Fig. 4. (A) FACS quantification (a.u.) of BODIPY-ceramide-labeled-exosomes released either by WT, RBL PLD2+, or RBL PLD2- cells. Means of 2–3 experiments  $\pm$  S.E.M. are presented. (B) C<sup>14</sup>-serotonin release by resting or ionomycin-activated Wt or PLD2+ RBL cells according to time. Typical experiment is presented.

results [15], was reported in Fig. 3A. In order to quantify the entire population of exosomes independently of any protein marker, we first labeled them with fluorescent lipids. We tested three fluorescent lipids and observed that the neutral lipid BODIPY-ceramide was the best marker of exosome membranes (Fig. 3B). Then, labeled exosomes were adsorbed on 3  $\mu$ M latex beads to be detected by FACS. As shown in Fig. 3C, monomeric beads (gate A) were mainly represented (74%, inset) and FACS analyses were restricted to this population.

As shown in Fig. 4A, PLD2 activity was clearly involved in exosome release, since about two times more exosomes were recovered from  $4 \times 10^6$  RBL PLD2+ and two times less from  $4 \times 10^6$  RBL PLD2- cells. As expected [6], RBL PLD2+ cells secreted two times more serotonin than Wt cells (Fig. 4B).

#### 4. Discussion

RBL-2H3 cells contain secretory granules corresponding to MVB [15], able to release upon stimulation their internal vesicles, called exosomes. Since exosomes trigger immune antitumoral response [13], it is of interest to understand the molecular mechanisms involved in their biogenesis and secretion. Different steps of secretion and intracellular trafficking require fusogenic lipids such as PA produced by PLD.

In the present work, we have observed a net enrichment of an active PLD2 isoform on exosomes. Interestingly, PLD2 undergoes intracellular redistribution from the plasma membrane (Fig. 1) upon cell stimulation. A possible PLD2 translocation from plasma membrane to secretory granules and then to exosomes during cell stimulation might account for exosome enrichment in this enzyme. Noteworthy, PLD2 internalization from plasma membrane towards intracellular vesicles has been previously reported [17]. Contamination of exosome preparations by plasma membrane vesicles can be ruled out, since exosomes purified in the same way displayed a characteristic lipid composition distinct from that of plasma membrane-derived vesicles [14].

In order to measure the amount of exosome released, we have adapted a previously described method [15]. In our case, exosomes were labeled with fluorescent lipids instead of fluorescent antibodies [15]. Short-chain BODIPY-ceramide, a neutral lipid, was the best tool to label exosome membranes as compared with NBD-PC and BODIPY-PC (Fig. 3B). This could be due to the impossibility of NBD-PC, contrarily to BODIPY-ceramide, to penetrate into liquid-ordered membrane domains with high lateral phospholipid pressure [18,19], like in exosome membranes [14]. In the same way, intracellular organelles are differently labeled with fluorescent lipids depending on their membrane lipid composition and fluidity [20].

As observed in Fig. 4A, overexpression of active or inactive PLD2 increases or decreases, respectively, exosome release. Involvement of PLD activity in this process could be related to the amount of secretory granules, which have fused with the plasma membrane. Indeed, PLD2 has already been shown to regulate secretion in RBL-2H3 cells [6]. However, PLD2 could also participate exosome biogenesis since in syk deficient B cells, with impaired PLD activation, MVB cannot be filled with exosomes [21].

In summary, the present work is the first report of an active signaling enzyme present on exosomes. Retrospectively, exosomal PLD could account for the noticeable extracellular PLD activity observed after activation of some cells, such as neutrophils [22] or fibroblasts [23]. Finally, PLD activity carried out by exosomes could be involved either in putative signaling properties of exosomes through second messengers such as PA, or in interaction mechanisms between exosomes and target cells through the fusogenic properties of PA. Exosomes may thus appear as a new signaling device.

*Acknowledgements:* Part of this work was supported by the ARECA Program. K. Laulagnier was supported by Association pour la Recherche contre le Cancer.

#### References

- [1] Liscovitch, M., Czarny, M., Fiucci, G. and Tang, X. (2000) *Biochem. J.* 345 (Pt 3), 401–415.
- [2] Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A. and Chen, J. (2001) *Science* 294, 1942–1945.
- [3] Blackwood, R.A., Smolen, J.E., Transue, A., Hessler, R.J., Harsh, D.M., Brower, R.C. and French, S. (1997) *Am. J. Physiol.* 272, C1279–C1285.
- [4] Burger, K.N. (2000) *Traffic* 1, 605–613.
- [5] Fensome, A., Cunningham, E., Prosser, S., Tan, S.K., Swigart, P., Thomas, G., Hsuan, J. and Cockcroft, S. (1996) *Curr. Biol.* 6, 730–738.
- [6] Choi, W.S., Kim, Y.M., Combs, C., Frohman, M.A. and Beaven, M.A. (2002) *J. Immunol.* 168, 5682–5689.
- [7] Chahdi, A., Choi, W.S., Kim, Y.M., Fraundorfer, P.F. and Beaven, M.A. (2002) *Mol. Immunol.* 38, 1269–1276.
- [8] Jenco, J.M., Rawlingson, A., Daniels, B. and Morris, A.J. (1998) *Biochemistry* 37, 4901–4909.
- [9] Lee, S., Park, J.B., Kim, J.H., Kim, Y., Shin, K.J., Lee, J.S., Ha, S.H., Suh, P.G. and Ryu, S.H. (2001) *J. Biol. Chem.* 276, 28252–28260.
- [10] Freyberg, Z., Sweeney, D., Siddhanta, A., Bourgoin, S., Frohman, M. and Shields, D. (2001) *Mol. Biol. Cell* 12, 943–955.
- [11] Freyberg, Z., Bourgoin, S. and Shields, D. (2002) *Mol. Biol. Cell* 13, 3930–3942.
- [12] Denzer, K., Kleijmeer, M.J., Heijnen, H.F., Stoorvogel, W. and Geuze, H.J. (2000) *J. Cell Sci.* 113 (Pt 19), 3365–3374.
- [13] Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Ricciardi-Castagnoli, P., Raposo, G. and Amigorena, S. (1998) *Nat. Med.* 4, 594–600.
- [14] Laulagnier, K., Motta, C., Hamdi, S., Roy, S., Fauvelle, F., Pageaux, J.F., Kobayashi, T., Salles, J.P., Perret, B., Bonnerot, C. and Record, M. (2004) *Biochem. J.* 380, 161–171.
- [15] Vincent-Schneider, H., Stumptner-Cuvelette, P., Lankar, D., Pain, S., Raposo, G., Benaroch, P. and Bonnerot, C. (2002) *Int. Immunol.* 14, 713–722.
- [16] Kemken, D., Mier, K., Katus, H.A., Richardt, G. and Kurz, T. (2000) *Anal. Biochem.* 286, 277–281.
- [17] Sciorra, V.A., Rudge, S.A., Wang, J., McLaughlin, S., Engebrecht, J. and Morris, A.J. (2002) *J. Cell Biol.* 159, 1039–1049.
- [18] Gidwani, A., Holowka, D. and Baird, B. (2001) *Biochemistry* 40, 12422–12429.
- [19] Gidwani, A., Brown, H.A., Holowka, D. and Baird, B. (2003) *J. Cell Sci.* 116, 3177–3187.
- [20] Mukherjee, S. and Maxfield, F.R. (2000) *Traffic* 1, 203–211.
- [21] Lankar, D., Vincent-Schneider, H., Briken, V., Yokozeki, T., Raposo, G. and Bonnerot, C. (2002) *J. Exp. Med.* 195, 461–472.
- [22] Tronchere, H., Planat, V., Record, M., Terce, F., Ribbes, G. and Chap, H. (1995) *J. Biol. Chem.* 270, 13138–13146.
- [23] Thorsen, V.A., Bruland, O., Lillehaug, J.R. and Holmsen, H. (1998) *Mol. Cell Biochem.* 187, 147–154.