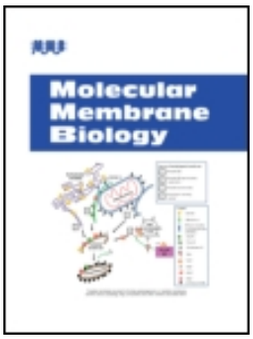

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Prothymosin alpha-receptor associates with lipid rafts in PHA-stimulated lymphocytes

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Abstract
Lipid rafts are specialized plasma membrane microdomains in which glycosphingolipids and cholesterol are major structural components. Their relative insolubility to nonionic detergents is the most widely used method to purify these structures. Several signalling proteins are associated with these microdomains in T lymphocytes, including receptors for growth factors and cytokines. ProTα is a highly conserved and widely distributed protein whose physiological functions remain elusive. In previous works we identified, by means of affinity cross-linking, affinity chromatography and fluorescence microscopy, a set of binding proteins for ProTα in human lymphoblasts. Now, this work goes deeply in that ProTα receptor description revealing, by different experimental approaches, its presence in lipid rafts. Moreover, our results fit a model in which a tyrosine phosphorylation signalling cascade confined to rafts is initiated upon ProTα receptor recognition, which represents an important and promising finding in the research for elucidating the molecular mechanisms underlying the immunomodulatory functions of ProTα.

Keywords: *Prothymosin α, rafts microdomains, confocal microscopy, phosphotyrosine pathway*

Abbreviations: MβCD, methyl beta cyclodextrin; ProTα, Prothymosin α; GPI, glycosylphosphatidylinositol; TX-100, Triton X-100; CTB, cholerae toxin B subunit; HRP, horseradish peroxidase; PE, phycoerythrin; FITC, fluorescein isothiocyanate; DAB, 3,3′-diaminobenzidine; GM1, ganglioside M1; mAb, monoclonal antibody; PHA, phytohemagglutinin; AP, alkaline phosphatase.

Introduction

The current view of the three-dimensional plasma membrane organization includes specific interactions between membrane lipids and proteins, producing a physical and functional compartmentation in dynamic microdomains also called rafts [1,2]. Rafts are enriched in extracellular GPI-anchored as well as cytoplasmic acylated proteins. However, transmembrane receptors or cytoskeletal proteins have been also detected by different means [3]. These microdomains deeply affect membrane functionality since the clustering of all these proteins (and associated molecules) in a very small area renders supramolecular complexes with new functions related with vesicular trafficking, signal transduction, viral gemation and cell motility [4–6]. However, despite the major advances made, the presence of rafts *in vivo*, their dynamics and stability as well as their actual dimensions are still under discussion [7–10].

The raft model has received much attention after the discovery of their role in the functionality of the lymphocyte, the central cell of the Immune System. This is because a crucial event in its biology, the signalling through the TCR/BCR receptors, is closely related with these membrane structures. According to a recent model both TCR and BCR are weakly associated with or even excluded from rafts during the resting state, and only upon triggering of these receptors (for example, after antibody-crosslinking) they move inside these microdomains [11–15], where Src kinases accumulate. The number of signalling processes related with proliferation and differentiation where it was found a link to rafts is increasing daily. Many cytokine and growth factor

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receptors are located in this region, including IL-2α [16], EGFR [17], PDGF [18], FGF [19] and insulin [20]. The current goal is not only to find out the signalling mechanisms triggered by these structures, but also the functional consequences that a membrane compartmentation could have on these transduction pathways.

Prothymosin α (ProTα) is a highly acidic and small protein of only 109 amino acids with an unusual primary structure. Based on its wide distribution or high conservation degree amongst mammals one would expect ProTα to play an essential role in the organism [21]. However, despite the number of effects described for this protein (e.g., intracellular modulator of nuclear processes [22], inhibitor of apoptosome formation [23] or extracellular functions; reviewed in Piñeiro et al. [21]) none of them have been really accepted as its actual physiological role.

The immunoregulatory properties of ProTα have been described both *in vivo* and *in vitro.* Perhaps, the most outstanding *in vivo* assays are the ones showing an anticancer activity for ProTα in an experimental tumour model, prolonging the survival of DBA/2 mice inoculated intraperitoneally with syngenic L1210 leukemic cells [24,25]. *In vitro*, ProTα has been shown to increase allo- and auto-mixed lymphocyte responses in multiple sclerosis and systemic lupus erythematosus patients [26,27]. Likewise, ProTα regulates IL-2 and PGE_2 secretion in bulk mononuclear cell-mediated lympholytic activities in cancer patients [28]. These effects are also observed in normal donors, where there is an enhancement of the PHA-induced proliferation of PBMC and an increased NK activity [29–33]. Finally, Eckert et al. opened the way for clinical applications of ProTα in melanoma and colorectal tumour patients [34,35].

Taking into account all the above mentioned biological responses to ProTα and after detecting a number of binding sites on the plasma membrane of lymphoid cells [36–38] our group started a study to broach the ProTα receptor characterization. As a result, affinity crosslinking and affinity chromatography experiments uncovered the existence of three binding proteins for ProTα of 31, 29 and 19 kDa on the cell surface of human lymphoblasts [39].

On the other hand, a basic criterion that a putative receptor for a specific ligand should met is that it must be present not only in all tissues and cell types showing pharmacological activity, but also located in a membrane region where adequately carry out its activity. In this report we demonstrate that different ProTα binding proteins (ProTα receptor) are detected on rafts from human lymphocytes and also that, upon ProTα receptor recognition by its ligand, there is a change in the tyrosine phosphorylation pattern of proteins exclusively located in rafts. Thus, our data point toward a ProTα receptor being responsible for the activities described for its ligand on the Immune System and reinforce the information gathered during the last years suggesting that the main part of the signalling processes related with cell proliferation and differentiation arise in these lipid rafts microdomains.

Material and methods

Cell isolation and culture

Buffy coats were kindly provided by the Centro de Transfusiones de Galicia, Santiago de Compostela, Spain. Human PBMCs (Peripheral Blood Mononuclear Cells) were isolated by Ficoll Paque PLUS (Amersham Biosciences) density gradient centrifugation as described [39]. Cells were cultured at 1×10^6 PBMCs/ml in RPMI 1640 (Sigma, Spain) supplemented with 10% inactivated FBS (Invitrogen, Spain), 100 μg/ml streptomycin and 100 IU/ml penicillin (Sigma), in a humidified atmosphere with 5% CO_2 at 37°C and activated with 2.5 μg/ml PHA-P (Sigma) [39]. After three days of culture, lymphoblasts (more than 90% $CD3^+$ T cells) were collected. Cell viability, assessed by trypan blue exclusion and flow cytometry analysis, was always higher than 90%.

Biotinylation

ProTα isolated from calf thymus and tested for purity by amino acid analysis and high voltage electrophoresis was kindly provided by Thymoorgan GmbH (Vieneenburg, Germany). ProTα (1 mg) was dissolved in 1 ml 0.1 M bicarbonate buffer pH 8.3 and subsequently conjugated with biotin-succinimidyl ester as previously described by Piñeiro et al. [39].

Isolation of TX-100 resistant membranes/rafts by equilibrium density gradient centrifugation

All the following steps were carried out at 4°C unless indicated and basically as described in Ilangumaran et al. [40–42]. PHA-lymphoblasts (50×10^6) were washed twice in PBS pH 7.4 and once in TKM buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 1 mM EDTA). Detergent lysates were prepared in TKM containing 0.5% Triton X-100 (TX-100) and the protease inhibitors Pefabloc SC (Roche Diagnotics, Barcelona; 2 mM), leupeptin (Sigma; 10 μg/ml) and aprotinin (Sigma; 5 μg/ml) for 20 min on ice. For equilibrium gradient centrifugation, cell extracts were adjusted to 40% sucrose and loaded into SW55Ti (Beckman L8-M) tubes.

Next, 2.7 ml of 36% sucrose and finally 1.575 ml of 5% sucrose, both solutions prepared in TKM buffer, were successively added. After centrifugation at 200,000 × g for 18 h, 450 μl fractions were collected from top to bottom, numbered (1–11) and stored at −20°C. Proteins of discontinuous gradient density fractions were evaluated by dot-blot immunoassay.

Affinity chromatography

The affinity matrix was prepared by coupling ProTα to a 1 ml NHS-activated HiTrap column (Amersham-Biosciences Europe GmbH, Barcelona, Spain). To this end, 1 ml of 0.2 M $NaHCO_3$, 0.5M NaCl pH 8.3 (coupling buffer) containing 6 mg of ProTα was injected into the column and the incubation was carried out for 4 h at 4°C. According to the manufacturer's instructions after the incubation the amount of protein bound to the column was determined by a BCA test. Several wash steps with buffers A (0.5 M ethanolamine, 0.5 M NaCl pH 8.3) and B (0.1 M acetate, 0.5 M NaCl pH 4) were performed in order to block any remaining active groups and remove the ligand excess. Column was equilibrated with TNE buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA) and coupled to an ÄKTA Purifier 10 (Amersham-Biosciences Europe GmbH, Barcelona, Spain) under the control of UNICORN 3.00 software. TX-100 resistant membranes (rafts) from PHA-activated lymphocytes were purified by equilibrium density gradient ultracentrifugation as described before, pooled and dialysed against TNE buffer. Samples (10–12 ml) was injected stepwise (2 ml each) into the column with a 0.5 ml/min flux. Non specific proteins were washed away with 30 ml of TNE buffer and elution was carried out with 0.1 M glycine pH 2.5 (0.5 ml/min flux). 1 ml fractions were collected, neutralised with 1 M Tris pH 9, concentrated (centricon-10, Millipore Corp., Bedford, MA, USA) and analysed by SDS-PAGE and silver staining.

Affinity cross-linking

Aliquots of 2×10^6 lymphoblasts were incubated with 40 μg biotin-ProTα at room temperature for 30 min. Chemical cross-linking was carried out in a 20 μl volume containing 1 mM BS^3 (Pierce Biotechnology, Inc, Rockford, USA) for 30 min at 4°C. Reaction was stopped by the addition of 1 ml TES buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 250 mM sucrose) and cells extensively washed in ice-cold PBS pH 7.4.

Immunostaining and immunofluorescence

Cells were cross-linked with biotin-ProTα and stained with streptavidin-PE and/or anti CD59-FITC (clon p282; BD-Biosciences, Madrid) and anti CD71-FITC (clon M-A712; BD-Biosciences). The percentage of cells positive for the Ag was evaluated by setting proper negative controls. For studies of detergent resistant proteins associated with rafts we have adapted the work of Janes et al. [43] to flow cytometry. Briefly, cells were treated with 1% TX-100 for 5 min on ice, 10 mM methyl-β-cyclodextrin (MβCD) (Sigma) for 15 min at 37°C, or MβCD followed by TX-100 extraction, before fixation (3% paraformaldehyde-60 mM sucrose in PBS pH 7.4 for 30 min at RT). Subsequently, cells were washed with PBS-20 mM glycine, blocked with PBS 5% BSA and stained as described above. Samples were processed on a Becton Dickinson FACScalibur flow cytometer and WinMDI software (a kind gift of J. Trotter, Scripps Institute, La Jolla, CA) was used to analyse the data.

Protein-depletion from rafts with CTB-HRP/DAB/H_2O_2

Depletion of microdomain-linked proteins was carried out, with modifications, according to Cheng et al. [44,45]. After a 3-day culture period with 2.5 μg/ml PHA human lymphoblasts were washed twice in ice-cold modified $HBSS^+$ (13 mM $CaCl_2$, 50 mM KCl, 5 mM $MgCl_2$, 4 mM $MgSO_4$, 1.38 M NaCl, 56 mM glucose and 200 mM Hepes, pH 7.4). Aliquots of 2×10^6 cells were incubated with cholera toxin B subunit (CTB; 0.01, 0.05, 0.1 or 0.1 μg/ml; Sigma) for 30 min at 37°C and washed with ice-cold $HBSS^+$. Samples were then resuspended at 4°C in 1 ml $HBSS^+$ containing diaminobencidine (DAB, Sigma; 0.5 mg/ml or 0.1 mg/ml), in the absence or presence of H_2O_2 (0.01, 0.05, or 0.1%), for 45 min. After two washes with cold $HBSS^+$, cell lysis was performed for 30 min on ice with TKM/0.5%TX-100/protease inhibitors and nuclei, debris and DBA cross-linked proteins eliminated after centrifugation at 13,000 rpm for 15 min (4°C). Once all the conditions for this protocol were set up aliquots of 2×10^6 lymphoblasts, with or without biotin-ProTα (15 μM) bound to the cell surface receptors with BS^3, were treated with CTB (0.05 μg/ml) and incubated in the absence or presence of DAB (0.5 mg/ml) and H_2O_2 (0.01%) as indicated.

Dot blot analysis

For these experiments two kinds of serially diluted samples were handled: (a) postnuclear lysates, nor-

malized for total protein or for an even number of cells, from biotin-ProTα (15 μM) labelled lymphoblasts, where rafts microdomains were depleted with the CTB-HRP based method described above; (b) equal volumes of samples from discontinuous density gradient fractions. Proteins were applied to the wells of a dot-blot apparatus (Bio-Rad Laboratories, Inc, CA, USA) to be transferred to nitrocellulose filters (Hybond^{ECL}, Amersham) for analysis with appropriate antibodies: anti CD59 (clon p282; BD-Biosciences), anti CD71 (clon M-A712; BD-Biosciences) or anti biotin-HRP (Sigma). Goat anti mouse (GAM)-HRP (Sigma) was used as a secondary Ab to reveal anti CD59 or anti CD71 binding to the membranes. Detection was carried out by using a nonisotopic chemiluminescent system (ECL$^+$, Amersham Biosciences) in all cases. When measuring alkaline phosphatase (AP) levels bromochloroindolyl phosphate/nitro blue tetrazolium was used as a substrate (BCIP/NBT; BioRad Laboratories). Spots were quantified by scanning the filters and densitometry (ImageMaster ID, Amersham Biosciences Europe GmbH).

Confocal microscopy

For confocal microscopy, aliquots of 2×10^6 PHA activated lymphoblasts were cross-linked with 1 mM BS^3 in the presence or absence of 15 μM biotin-ProTα. Cells were incubated with streptavidin-PE to detect ProTα receptor expression and stained with anti CD59 or anti CD71 mAbs at 4°C. In order to induce either rafts (with anti-CD59) or non-raft (with anti-CD71) protein clustering and detect both CD59 and CD71 antigens, a further incubation step at RT (i.e., allowing free lateral movements) with a FITC-labelled anti-IgG Ab was performed. After washing with PBS pH 7.4 and SlowFade equilibrium buffer, cells were finally resuspended in a antifading Slowfade solution (Molecular Probes, Inc., OR, USA). Observations were made with a Leica TCS 4D confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope (Leica Lasertechnik GmbH, Heidelberg, Germany). Colocalization analysis was performed by means of the MultiColour software (version 2.0; Leica Lasertechnik GmbH).

Western blotting

To detect tyrosine phosphorylation of proteins in total cell lysates, human lymphoblasts (5×10^6) were placed in 100 μl of RPMI-1640 and incubated at 37°C for 10 min. Then, two 5 μl aliquots (controls) were collected and the rest was divided in two identical (2×10^6 cells) samples that were treated with 45 μl of RPMI-1640 containing or not ProTα (5 μg/ml final concentration). 10 μl samples were removed at different times and cellular lysis was carried out immediately by the addition of an equal volume of 2× SDS-PAGE Laemmli sample buffer. In another set of experiments, lymphoblasts (50×10^6) obtained as above and treated or not for 1 min at 37°C with 5 μg/ml of ProTα were subjected to equilibrium density gradient centrifugation as previously indicated. Insoluble (3–6) and soluble (10–11) fractions were pooled, protein concentration of every sample determined and either the same volume or the same amount of protein loaded in a 7.5% SDS-PAGE. After transferring to PVDF (Hybond^P) or nitrocellulose (Hybond^{ECL}) membranes (Amersham Biosciences Europe GmbH) blots were blocked with 1% BSA in TBS-T (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween-20) 1 h at RT, hybridized with anti phosphotyrosine (clon PY20, BD-Biosciences) in blocking buffer 30 min at RT, washed extensively with TBS-T and incubated with GAM-HRP under the same conditions. Bands were detected using ECL$^+$ and revealed with X-OMAT XAR 5 (Eastman-Kodak, Sigma).

Results

ProTα receptor isolated from detergent-resistant membrane fractions

We recently demonstrated by fluorescence microscopy and biotin-ProTα cross-linking to human lymphoblasts that there is a set of ProTα binding proteins heterogeneously distributed on the cell surface to form a cap at one of the poles [39]. Taking into account that the same capping phenomenon has been described for many important receptors we came to the conclusion that a study on the membrane distribution of this receptor would be interesting. Therefore, PHA-activated lymphocytes were cultured and lipid rafts isolated based on their nonionic detergent insolubility at low temperature and their density when submitted to a discontinuous sucrose gradient ultracentrifugation step. Eleven fractions, collected and numbered from the top to the bottom of the tube, were analysed for protein concentration and AP activity (Figure 1A) in order to be confident about the conditions used in our raft purification protocol [46,47].

Next, we carried out the same kind of experiments but using human lymphoblasts cross-linked with biotin-ProTα accordingly with the protocol we had previously described [39]. Analysis of those cells by flow cytometry showed activated lymphoblasts exhibiting the correct cell morphology and being, in

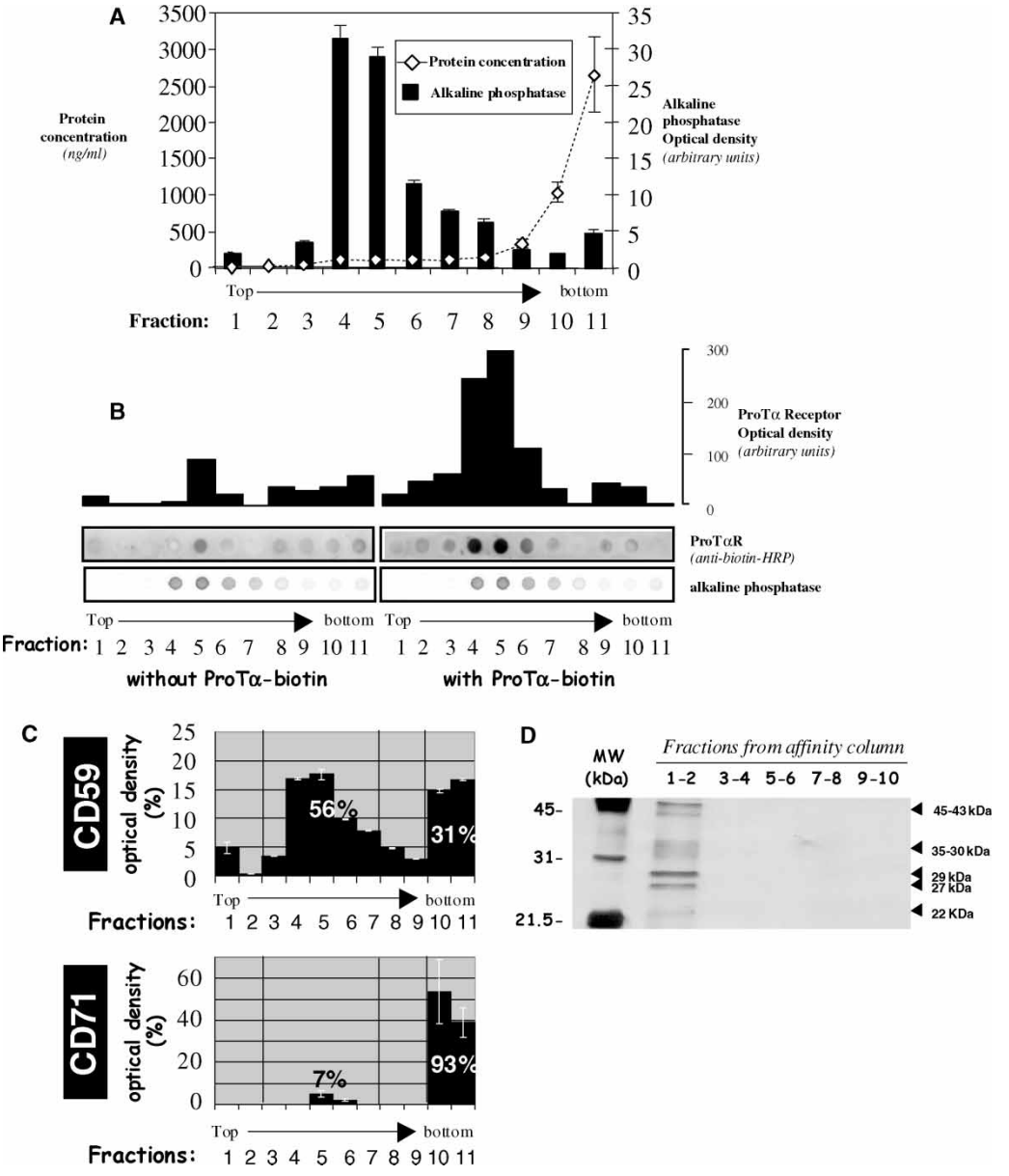

Figure 1. ProTα receptor is located in rafts microdomains. After BS^3-mediated crosslinking of biotin-ProTα to surface receptors on human lymphoblasts (50×10^6) cell lysis was carried out in TKM buffer containing 0.5% TX-100. The cell lysate was then adjusted to 40% sucrose and subjected to equilibrium density gradient centrifugation in a SW55Ti rotor. After a overnight centrifugation at 200,000 ×g, 11 fractions (0.45 ml/each) were collected from top to bottom. Serial dilutions of the fractions were dotted on and analysed for total protein (BCA assay; a), alkaline phosphatase activity (BCIP/NBT-based assay; a and b), and the presence of ProTα receptor (b), CD59 (c) and CD71 (c). Films were scanned, subjected to densitometry, and data shown as raw arbitrary units (b) or expressed as a percentage of the total amount for the respective antigen (a, c). (d) Analysis by SDS-PAGE of pooled fractions obtained after running a raft membrane extract from human lymphoblasts through a ProTα affinity column.

a vast majority (>90%), positive for the ProTα receptor (data not shown). To study the presence of the receptor in membrane fractions isolated by discontinuous sucrose density gradient ultracentrifugation, dot-blot analyses were performed. We used in this set of experiments peroxidase-conjugated monoclonal antibody against biotin. As observed in Figure 1B, most of the labelling from anti biotin-antibody was detectable on fractions 4–6, the ones with the highest levels of AP activity. We also analysed (Figure 1C) the presence of both CD59 (as raft marker) and CD71 (a protein normally excluded from rafts) in order to attain the highest stringent conditions when purifying the different membrane fractions. These controls also pointed out to the 4–6 fractions as the ones containing exclusively raft resident proteins. On the other hand, experiments designed to characterize and purify the Prothymosin α receptor (manuscript in preparation) allowed us to detect in rafts, by affinity

chromatography, several protein species of about 35–30, 29–27 and 22–19 kDa (Figure 1D), providing strong evidences about a ProTα interaction with proteins located within lipid rafts.

ProTα receptor is present on lipid rafts from living cells

It has been described that the method based on the insolubility in nonionic detergents for the analysis of raft domains suffers from some technical limitations [48]. Taking that into account, we employed other techniques that could reflect, in a more reliable way, the actual situation of the ProTα receptor in living cells.

Cholera toxin B subunit (CTB) exhibits a great affinity for ganglioside GM1, a glycosphyngolipid present almost exclusively in raft regions [10]. Cell surface labelling with CTB linked to peroxidase (CTB-HRP) allows a specific cross-linking of surrounding proteins in the presence of hydrogen peroxide (H_2O_2) and 3,3′-diaminobenzidine (DAB). Any protein at close proximity to CTB-HRP will be polymerized into insoluble aggregates when DAB and H_2O_2 are both present, but not in the absence of H_2O_2. Hence, only proteins displayed within the area where GM1 is present *in vivo* will precipitate after cell lysis and centrifugation. This technique has been designed to work with closed cellular microenvironments, such as organelles or vesicles [49]. The adaptation to our experimental system was carried out using two well-established raft- and soluble- markers (CD59 and CD71, respectively) and different concentrations of CTB-HRP, DAB and H_2O_2 to induce chemical cross-linking. As observed in Figure 2, the different concentrations of reagents generate a differential drag of the components analysed, being some conditions so aggressive that eliminate even the transferrin receptor (CD71). On the contrary, other conditions were observed to be totally ineffective in eliminating CD59. Hence, the amounts of reagents considered to be optimal were 0.05 μg/ml CTB-HRP, 0.01% H_2O_2 and 0.5 mg/ml DAB (Figure 2, arrow).

The next step was to evaluate the capacity of the chemical cross-linking of proteins residing at the same microenvironment as GM1 to eliminate the ProTα receptor. Figure 3 clearly shows how after the chemical cross-linking and elimination of raft proteins induced by CTB-HRP, H_2O_2 and DAB both ProTα receptor (Figure 3A) and CD59 (Figure 3B) were no longer detectable in the sample. Besides, it is also observed how the signal of the samples treated with DAB in the absence of H_2O_2 is the same as the one of cells incubated exclusively with CTB-HRP. Taking into account all these results we can state that the ProTα receptor is associated to raft microdomains *in vivo* and not as a consequence of experimental manipulations or artefacts.

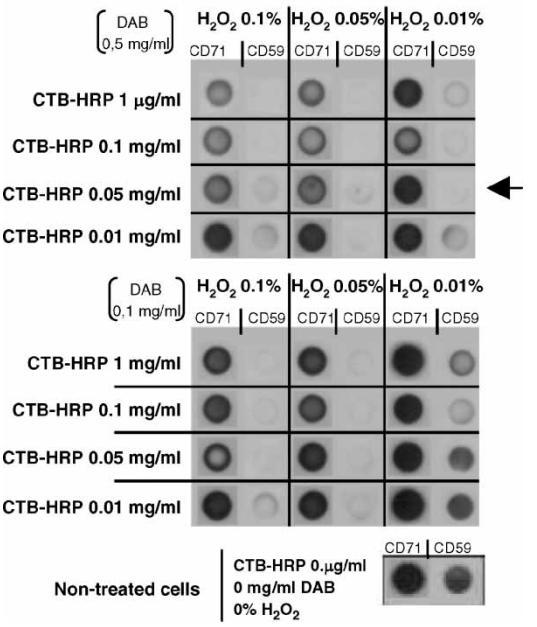

Figure 2. Improvement of a CTB-HRP-based method for the specific depletion of raft proteins. Lymphoblasts were separated in different samples, incubated with various amounts of cholera toxin (CTB; 0.01, 0.05, 0.1 or 1 μg/ml) for 30 min at 37°C and then washed several times with $HBSS^+$. Next, cells were placed in 1 ml $HBSS^+$ and the 3,3′-diaminobenzidine (DAB) crosslinker, either at 0.5 or 0.1 mg/ml, added in the presence of different concentrations of H_2O_2 (0.01, 0.05 or 0.1%) for 45 min at 4°C. After this incubation step, lymphoblasts were washed twice with cold $HBSS^+$ and cell lysis performed with TKM/0.5% TX-100 for 30 min on ice. A postnuclear supernatant was obtained after centrifugation at 13,000 rpm for 15 min (4°C) to eliminate, amongst others, nuclei and rafts proteins. Soluble proteins (non raft proteins) were dotted on a nitrocellulose membrane (Hybond^{ECL}, Amersham-Biosciences Europe, GmbH) and the presence of CD59 (raft marker) and CD71 (non raft marker) analysed as in Figure 1 by Immunoblotting combined with ECL^{Plus} (Amersham-Biosciences Europe, GmbH). Initial expression of both CD59 and CD71 was evaluated in non-treated cells.

ProTα receptor location corresponds to membrane areas highly resistant to MβCD and TX-100

The effect of cholesterol removal on rafts integrity was assessed by treating biotin-ProTα cross-linked lymphoblasts with either TX-100, a non ionic detergent which eliminates non-raft proteins, or MβCD (methyl β-cyclodextrin), which efficiently extracted cholesterol from cells. Figure 4 shows the results from a typical experiment obtained after biotin-ProTα cross-linking, detergent treatment and immunofluorescent detection with streptavidin-PE. It can be observed that none of the conditions eliminate the ProTα receptor labelling. To test the efficiency of the treatment a well-known TX-100 sensitive and MβCD-resistent cell surface marker, CD71, was also used (Figure 4), which allowed us to

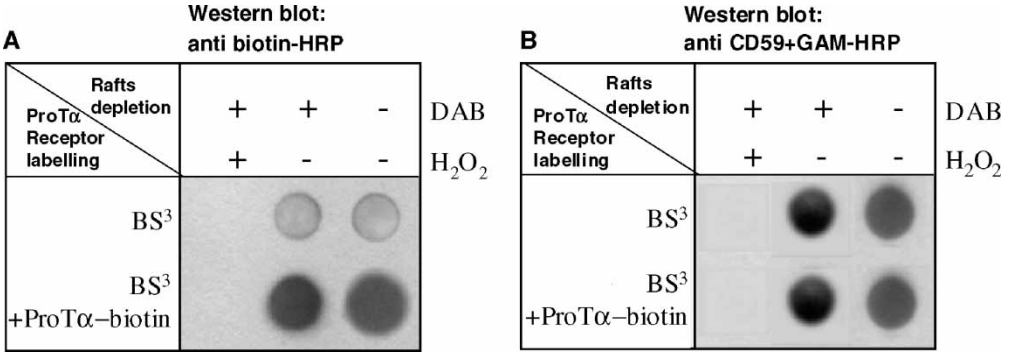

Figure 3. Raft protein crosslinking and depletion from lymphoblasts leads also to a ProTα receptor removal. Aliquots of 2×10^6 PHA-activated cells were cross-linked with 20 μl mM 1 BS^3, in the absence or presence of 15 μM biotin-ProTα. To eliminate raft proteins all samples were treated with CTB-HRP (0.05 μg/ml) and incubated (+) or not (−) with DAB (0.5 μg/ml) in the absence (−) or presence (+) of H_2O_2 (0.01%) as indicated. After lysis and centrifugation, samples were dotted on a nitrocellulose membrane and the presence of ProTα receptor (a) and CD59 (b) detected with anti biotin-HRP and anti CD59+GAM-HRP, respectively.

be sure about the experimental conditions. Once again we can conclude that our results are consistent with an *in vivo* ProTα receptor location within rafts.

If we observe the histogram profiles (Figure 5A) where TX-100 has been used to extract part of the lipid fraction of the membrane it easily follows that ProTα receptor signal is not reduced but increased. The effect of these treatments on cell size and microstructure of the plasma membrane was also analysed. Thus, cells treated with TX-100 showed a decrease in size due to the loss of a great part of their lipid content in plasma membrane (Figure 5B). But, at the same time, its structure likely presents a relaxation that could allow somehow the entrance of streptavidin-PE inside cells. Further controls, subjected to the same cross-linking treatment but in the absence of biotin-ProTα, allowed us to rule out a non-specific binding of streptavidin-PE to intracellular components as responsible for that enhanced receptor fluorescence (Figure 5A). Therefore, the most plausible explanation was that streptavidin-PE is detecting an internalized ProTα-receptor complex. In fact, the same kind of observation can be made with the GM1 ganglioside, a raft marker with important intracellular stores, detected by CTB-Alexa 488 immunofluorescent staining, and whose expression is enhanced upon TX-100 treatment (Figure 5C). Thus, these results gave more support to our hypothesis of internalization for the ProTα receptor [36–38], a phenomenon normally associated with signalling through growth factors receptors [50].

ProTα receptor is colocalized with aggregated rafts microdomains generated by surface cross-linking of CD59

To obtain confocal images showing the above mentioned ProTα receptor inclusion in raft regions from biotin-ProTα cross-linked lymphoblasts, we compared the ProTα receptor surface location with CD59 or CD71 distribution. In order to carry out these experiments either CD59 or CD71 antigens from biotin-ProTα cross-linked lymphoblasts were labelled with anti CD59-FITC or anti CD71-FITC and patching induced with a FITC-labelled anti IgG. To allow free lateral movements the last incubation was performed at room temperature.

As observed in Figure 6, both CD71 and CD59 markers present a distribution with a dotted appearance, which indicates that the antibody cross-linking was effective in generating aggregates. To determine the correspondence between the physical locations of these molecules on biotin-ProTα cross-linked lymphoblasts, double-immunofluorescence analyses were carried out with either anti CD59-FITC/Streptavidin-PE or anti CD71/Streptavidin-PE combinations. In the double labelled anti CD71-FITC/Streptavidin-PE samples the signal detected by confocal microscopy corresponded with separate areas of the membrane (Figure 6). However, anti CD59-FITC/Streptavidin-PE labelled PHA-stimulated lymphocytes showed that both CD59 and ProTα receptor molecules were colocalized in rafts (Figure 6). Furthermore, since these images have been taken using a 63 × objective, with a resolution limit for the z-axis of 0.23 μm, it is likely that both proteins, ProTα receptor and CD59, are confined in an area with a diameter $\leq$230 nm.

Increased tyrosine phosphorylation in response to ProTα takes place in raft microdomains from T lymphoblasts

Next we investigated the possible transduction mechanisms promoted by the interaction between ProTα and its receptor on PHA-lymphoblasts. As a first approach, whole lysates from cells treated with 5 μg/ml ProTα for various times were analysed,

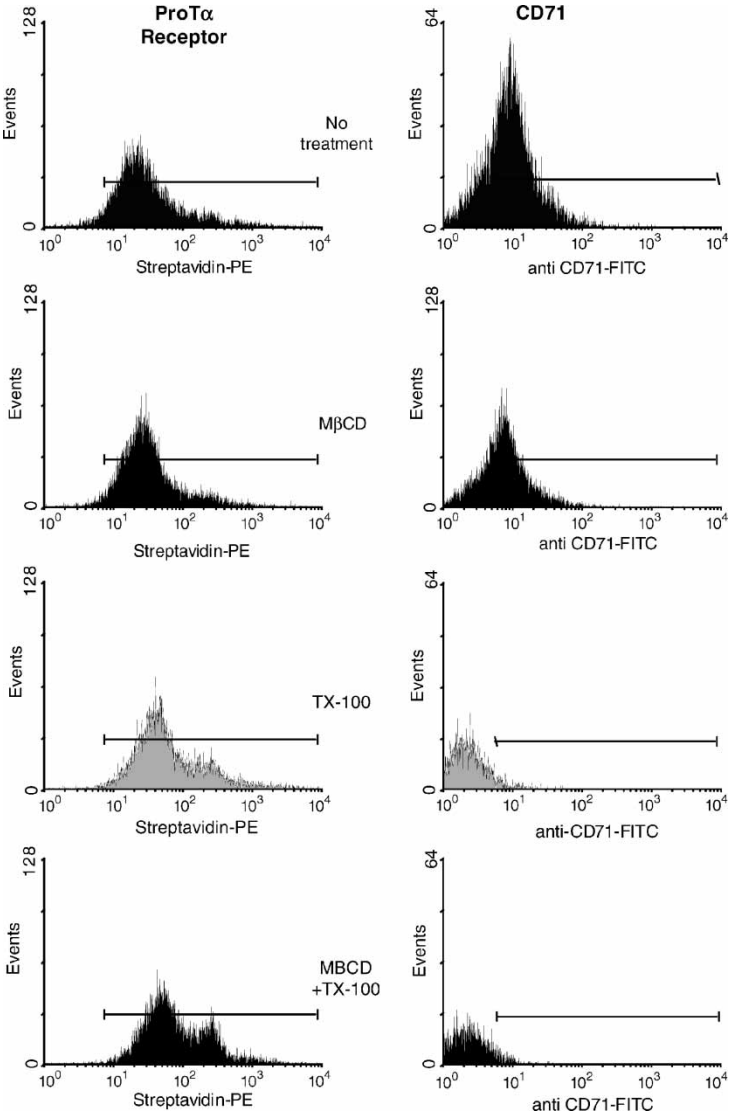

Figure 4. Flow cytometry analysis of the ProTα receptor association with raft regions on human lymphoblasts. Cells (2×10^6) were cross-linked with BS^3 in the absence (to check for background fluorescence; see also Figure 5a, right) or the presence of 15 μM of biotin-ProTα, and then treated with 1% TX-100, 10 mM MβCD or both as indicated in Material and Methods. After paraformaldehyde fixation, ProTα receptor and CD71 expression was revealed by staining with streptavidin-PE and anti CD71-FITC, respectively. In order to know the percentage of $CD71^+$ cells and to place the histogram marker as shown, a FITC-labelled $IgG_{2a}\kappa$ isotype antibody was used as a negative control. Data acquisition was done on a Becton Dickinson FACScalibur flow cytometer, while WinMDI software was used to analyse the data. This experiment is representative of several with similar results.

showing that ProTα strongly induced a transitory tyrosine phosphorylation of multiple proteins after 30 s incubation, with a maximum at 15 min (Figure 7A).

An additional support for the ProTα receptor presence in rafts microdomains was obtained when we evaluated the distribution of these tyrosine phosphorylated proteins among different membrane fractions. Cells were incubated or not for 1 min with 5 μg/ml ProTα and fractions from discontinuous sucrose density gradients, corresponding to either TX-100 insoluble (fractions 3–6; lipid rafts) or soluble membranes (fractions 10–11), pooled. Protein quantification of all the samples was carried out because of the substantial differences between raft and non-raft fractions and the very fast protein distribution we have observed upon one minute incubation with ProTα (Figure 7B), which could alter our perception about the magnitude of the tyrosine phosphorylation taking place. Based on that, we decided to carry out several experiments loading SDS-PAGE gels with either the same amount of membrane protein (Figure 7C) or identical fractions volumes (Figure 7D). In both cases, it was apparent an induced tyrosine phosphorylation in several rafts resident proteins from ProTα-treated

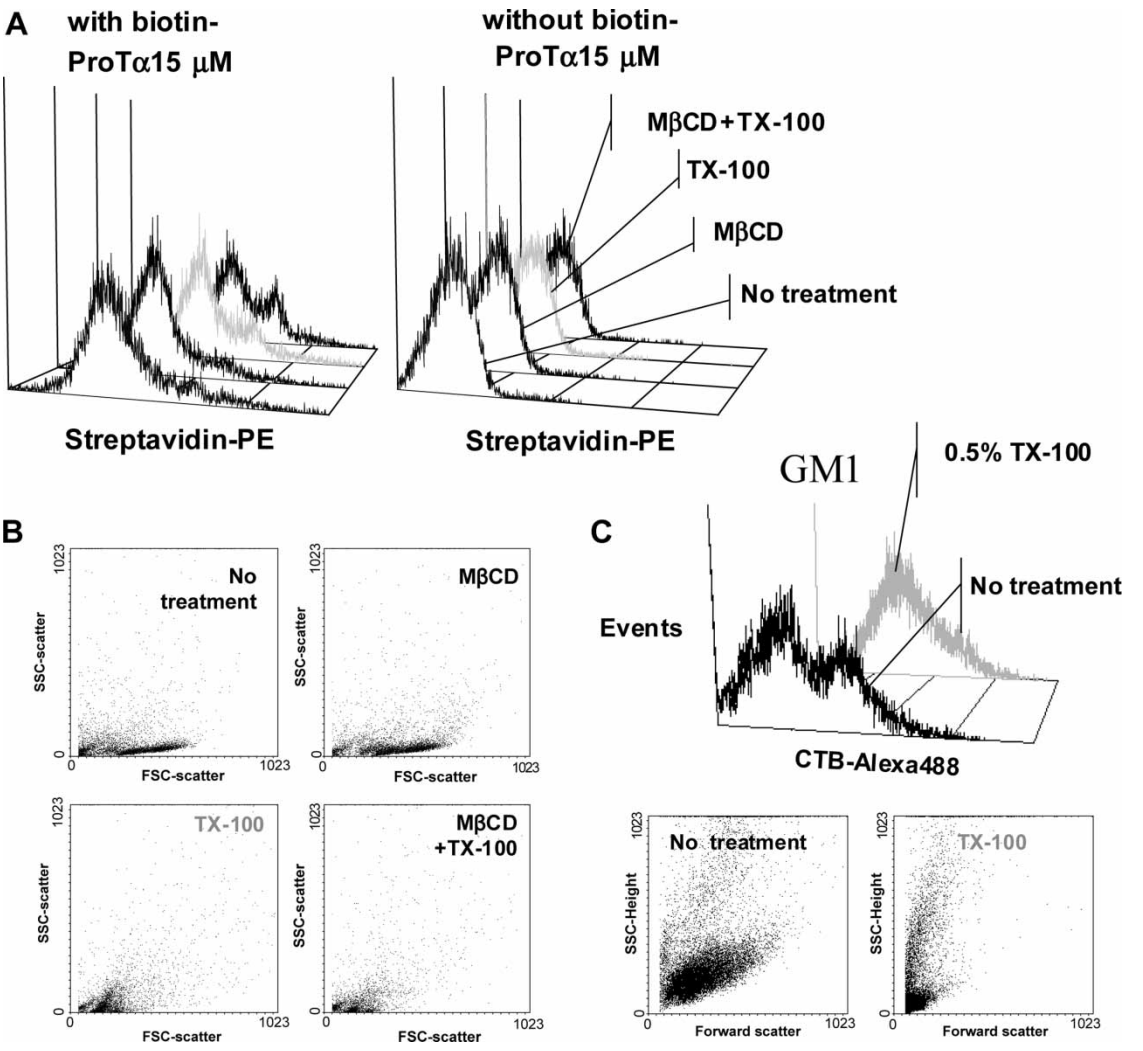

Figure 5. High resistance to non ionic detergent extraction is consistent with an *in vivo* location of ProTα receptor within rafts. Cells from samples described in Figure 4 were analysed by flow cytometry for streptavidin-PE (ProTα receptor) immunofluorescence (a) and for forward and right-angle light scattering (b). In (a), negative control cells (right) were not incubated with biotin-ProTα in order to show the non specific binding of streptavidin-PE. Dot plot (forward versus right-angle scattering) and histograms (CTB-Alexa 488 versus events) shown in figure (c) represent human PHA-lymphoblasts from a different donor treated with or without 1% TX-100 and stained with CTB-Alexa 488 to reveal GM1 ganglioside expression.

versus untreated cells, being even more evident when equal amounts of proteins were resolved by SDS-PAGE (Figure 7C). On the contrary, tyrosine phosphorylated species were hardly detected in TX-100-soluble membrane fractions, and only when the same sample volume was loaded (Figure 7D). We should mention that all these phosphorylated proteins (asterisks in Western blot of Figures 7C and D) were consistently present in raft fractions from ProTα-treated cells and were similar in size to some of the phosphorylated proteins detected in the whole cell lysates (Figure 7A). Therefore, our results definitively involve a tyrosine phosphorylation cascade taking place in a raft microdomain environment as a response to the interaction of ProTα with its receptor, which agree with our previous results showing a proliferative effect of ProTα [29].

Discussion

Due to all the *in vitro* and *in vivo* immunoregulatory properties previously shown by ProTα is of fundamental importance to elucidate the molecular mechanisms underlying these functions and, therefore, to understand the nature of the ProTα interaction with the cell surface. Recently, we demonstrated by affinity cross-linking and affinity chromatography the existence of binding sites for ProTα in human lymphoblasts, detecting also by fluorescence microscopy a heterogeneous distribution of this ProTα receptor in a cap-like structure at one of the cell poles [39]. To test whether this particular receptor polarization had also some type of relationship with a specific location in differentiated plasma membrane compartments we started

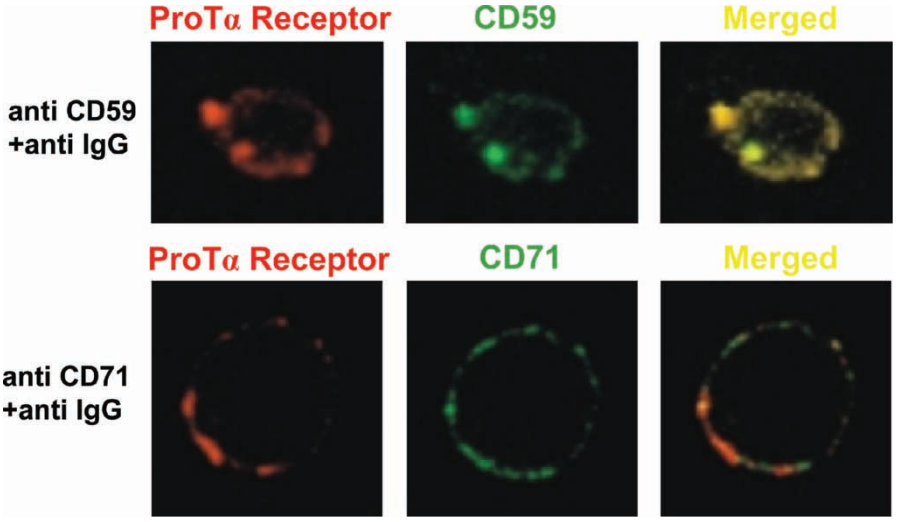

Figure 6. ProTα receptor and CD59 show a similar distribution pattern on plasma membrane from human lymphoblasts. Aliquots of 2×10^6 cells were cross-linked with 1 mM BS^3 in the presence (figure) or absence (not shown) of 15 μM biotin-ProTα. Lymphoblasts were incubated with streptavidin-PE to reveal ProTα receptor expression (red) and also, in order to induce either raft or non-raft proteins clustering, stained with anti CD59 or anti CD71 mAbs respectively at 4°C and incubated at RT (to allow free lateral movements of proteins) with a FITC-labelled anti-IgG Ab to cross-link any antibody on the cell surface. Both CD59 and CD71 expression are shown in green, while colocalization level (merged) of any of these markers with ProTα receptor is presented in yellow.

purifying, by discontinuous sucrose density gradients, both raft (TX-100 insoluble) and non-raft (TX-100 soluble) membrane fractions. In agreement with other authors [47] rafts proteins (7%) were detected in detergent insoluble fractions (3–6), while most of the protein (85%) appeared in the soluble area (10–11). ProTα receptor was found in strong association with raft microdomains, as well as several well-known raft proteins (alkaline phosphatase, CD59). Thus, both ProTα receptor and alkaline phosphatase (AP) showed a normal distribution between fractions 3–6 [46], while CD59, a GPI-anchored protein whose presence in rafts is prominent, revealed also a percentage of CD59 (31%) still present in non-raft fractions. This last finding could result from its weak association with these microdomains, the extraction procedure or the maturity degree of the protein (presence or not of the GPI-anchorage) [40]. On the contrary CD71, a transmembrane protein accessible for nonionic detergents, proved to be a good marker for non-raft membrane areas and showed a different membrane distribution when compared with ProTα receptor.

However, in spite of nonionic detergents insolubility and discontinuous density gradient ultracentrifugation have been shown to be a good starting point to detect raft proteins and several studies prove its validity [51–53], it has been also published that this technique presents some limitations [48]. Thus, it is currently under debate if data obtained with this purification method really reflects the actual *in vivo* situation or it is merely an artefact. As well, there are some reasonable doubts whether this technique is adequate for detecting raft proteins whose association to rafts is weak [40,43]. To sort all these problems out, we decided to use a different experimental approach. Cholera toxin B subunit (CTB) colocalization is equivalent to the association of the protein under study with rafts in intact cells [43]. Therefore, we wondered if, under very controlled conditions, the capacity of the peroxidase (HRP) linked to CTB to catalyze a massive protein cross-linking in the presence of DAB and H_2O_2 would delete ProTα receptor from cells. Once again, our results probed, in agreement with our data obtained from sucrose gradients, that both CD59 and ProTα receptor were located at the same raft microenvironment.

Cyclodextrins are non invasive tools of extracting cholesterol in cell membranes. Because cholesterol is essential for raft integrity [52] many proteins associated with these microdomains are extracted with MβCD or acquire sensitivity to solubilization with nonionic detergents after treatment with MβCD [40]. Thus, by using nonionic detergents and cyclodextrins prior to flow cytometry analysis, we wanted to test the exact position of ProTα receptor within the plasma membrane. The ProTα receptor labelling persistence upon TX-100 and/or MβCD treatment was consistent with its presence in rafts microdomains of human lymphoblasts.

Studies carried out by chemical cross-linking of markers [54] and FRET [55,56] have demonstrated that raft domains are dynamic structures whose mean diameter is around 70 nm in living cells. For such a reason they are outside the limits of resolution

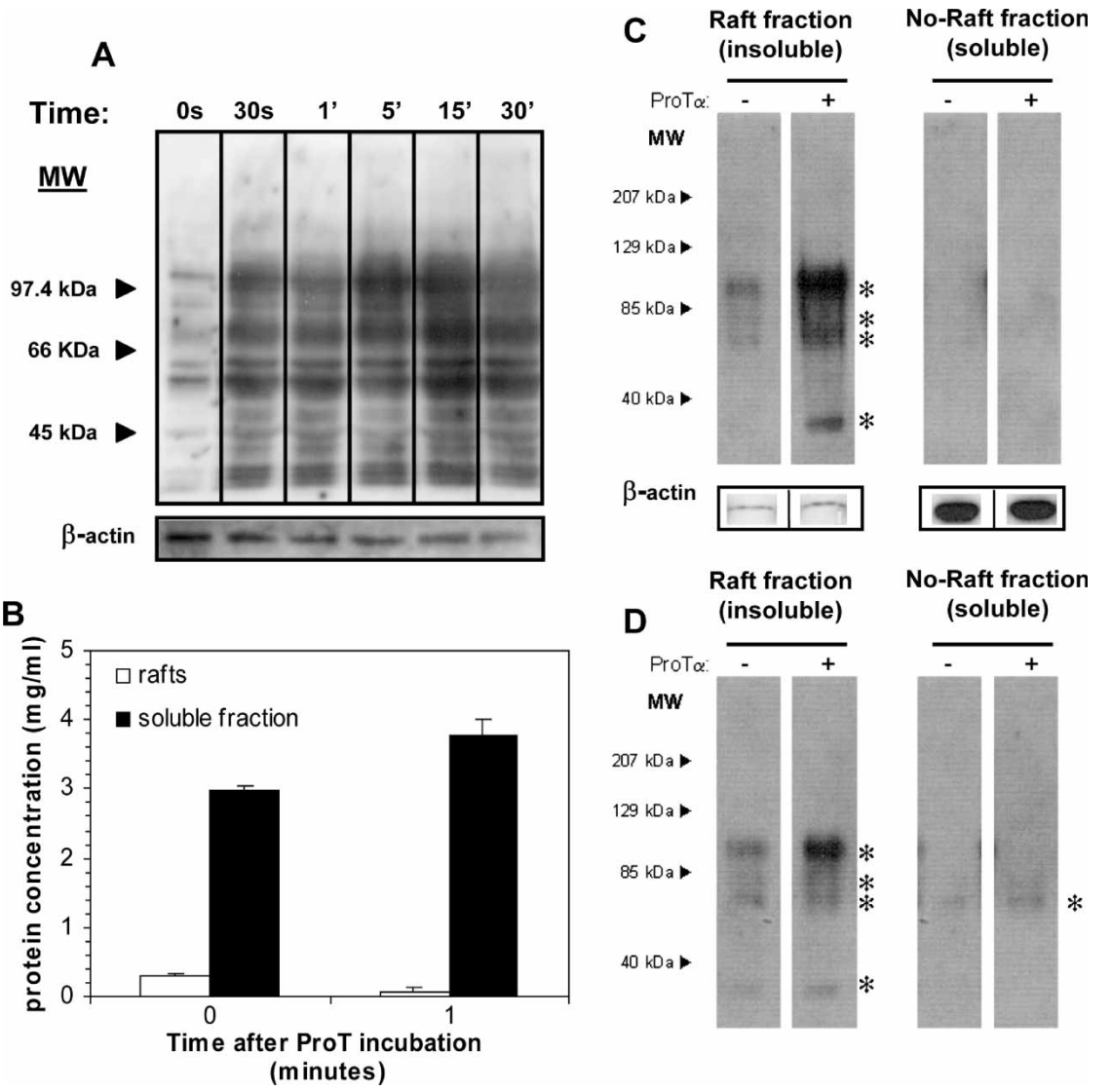

Figure 7. ProTα induces a tyrosine phosphorylation event confined to rafts in lymphoblasts. (a) Cells were incubated or not with 5 μg/ml of ProTα for different times and their lysates run on a 7.5% SDS-PAGE electrophoresis gel, transferred to a PVDF membrane and the presence of phosphotyrosines revealed with an anti-phosphotyrosine mAb (clone PY20, BD-Biosciences) in combination with GAM-HRP and ECL^{Plus} (Amersham Biosciences Europe, GmbH). (b) Proteins from cells incubated or not with 5 μg/ml of ProTα for 1 min at 37°C were extracted as indicated in Figure 1. Raft fractions (3–6) and soluble fractions (10–11) were pooled and protein concentration determined (BCA, Pierce Biotechnology, Inc). Identical amounts of protein (2 μg; c) and volume (10 μl; d) from both pooled insoluble and soluble fractions were run on a 7.5% SDS-PAGE gel and detection of phosphotyrosines performed as before. In a and c protein loading controls were established after membrane stripping and incubation with an anti β-actin mAb.

of conventional optical microscopy techniques. However, lipids and proteins exposed extracellularly can be laterally cross-linked by means of antibodies or multivalent bacterial toxins (e.g., CTB), which causes its aggregation on the cell surface [57]. Therefore, cross-linking of known raft components has allowed us to sort out the above mentioned problem and determine the suspected association of some proteins with rafts [43,58]. By using this approach ProTα receptor was also found to be close to CD59 in cholesterol/esfingolipids enriched membrane microdomains. However, it has been also described that many receptors migrate to a raft region only after binding to its specific ligand, and vice versa. Due to the fact that in our experiments the detection of the receptor by means of either confocal microscopy or flow cytometry can only be achieved after binding ProTα, its location should correspond to rafts when the receptor has been engaged. Therefore, purification and identification of all the acceptor molecules for ProTα with different techniques and strategies will allow us to clarify their location before or after the binding of a specific ligand. In this sense, affinity chromatography experiments carried out to characterize and purify the ProTα receptor led to the detection in rafts

preparations of several protein species of about 35–30, 29–27 and 22–19 kDa (the present data and manuscript in preparation). However, since it is known that not all the rafts proteins come from the lymphocyte plasma membrane [48 and our data] and ProTα seems to have both intracellular and extracellular functions [21], it is likely that not all these binding proteins are linked to the actual surface receptor. Studies are currently being carried out to gain insights on this regard.

As mentioned above, confocal microscopy provided us with data supporting the fact that both ProTα receptor and CD59 were close to each other when the former is aggregated by its ligand and probably located in an area (clustered rafts) with a diameter no bigger than 230 nm (the maximum distance to see colocalization between two proteins). Therefore, we have a receptor not only situated in cells which the immunoregulatory properties of ProTα have been described for, but also a receptor placed in a plasma membrane area (rafts) where many receptors known to have similar biological activities reside and where the signalling processes for differentiation and proliferation seem to be more active. In agreement with that we provided experimental evidence supporting the fact that a phosphotyrosine pathway is initiated upon ProTα receptor recognition and that such a signalling cascade is confined to rafts. It has been previously reported by other authors as well as ourselves that ProTα may act as a co-stimulator in the proliferation process [28–30]. Our data are relevant because they show a ProTα receptor and a ligand induced tyrosine phosphorylation located in rafts from human lymphoblasts. This allows us to feel more confident about the idea that this receptor possesses the necessary requirements to form part of the mechanism of action of ProTα as a biological response modifier in the Immune System. Therefore, an aspect that should be studied, as soon as the sequence become available, is the possible secondary modification responsible for its association to raft regions. The search for that modification on the receptor and the identification of all the participants in the signalling cascade triggered by ProTα will lead us to a better understanding at a molecular level of this peculiar protein.

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