Different glycoforms of prostate-specific membrane antigen are intracellularly transported through their association with distinct detergent-resistant membranes

Deborah CASTELLETTI*1, Marwan ALFALAH*1, Martin HEINE*1, Zeynep HEIN*, Ruth SCHMITTE*, Giulio FRACASSO†, Marco COLOMBATTI† and Hassan Y. NAIM†2

*Department of Physiological Chemistry, University of Veterinary Medicine Hannover, Bünteweg 17, D-30559 Hannover, Germany, and †Department of Pathology, University of Verona, Policlinico G.B. Rossi, P.le L.A. Scuro n.10, 37134 Italy

Hormone-refractory prostate carcinomas as well as the neovascularature of different tumours express high levels of PSMA (prostate-specific membrane antigen). PSMA is a type II transmembrane glycoprotein and a potential tumour marker for both diagnosis and passive immunotherapy. Here, we report on the association of PSMA with DRMs (detergent-resistant membranes) at different stages of the protein maturation pathway in human prostate carcinoma LNCaP cells. At least three PSMA glycoforms were biochemically identified based on their extractability behaviour in different non-ionic detergents. In particular, one precursor glycoform of PSMA is associated with Tween 20-insoluble DRMs, whereas the complex glycosylated protein segregates into membrane structures that are insoluble in Lubrol WX and display a different lipid composition. Association of PSMA with these membranes occurs in the Golgi compartment together with the acquisition of a native conformation. PSMA homodimers reach the plasma membrane of LNCaP cells in Lubrol WX-insoluble lipid/protein complexes. At the steady state, the majority of PSMA remains within these membrane microdomains at the cell surface. We conclude that the intracellular transport of PSMA occurs through populations of DRMs distinct for each biosynthetic form and cellular compartment.

Key words: cancer, detergent-resistant membranes, intracellular protein trafficking, membrane microdomains, prostate-specific membrane antigen (PSMA), secretory pathway.

INTRODUCTION

PSMA (prostate-specific membrane antigen) [1] was initially immunosolated from a lymph node metastasis of a prostate carcinoma [2]. Increased expression of PSMA in prostate cancer cells with respect to healthy tissues [3] and its de novo synthesis in the neovascularature of many solid tumours [4] render PSMA an attractive marker for prostate tumour diagnosis [5] as well as targeted immunotherapy. In particular, PSMA represents a very suitable target for antibody-vehicled drugs or immunotoxins [6] due to high levels of the protein at the cell surface and efficient antibody-mediated internalization [7].

PSMA is a type II transmembrane glycoprotein of approx. 100 kDa [8] that is transported to the apical membrane in polarized epithelial cells [9]. Its high level of N-glycosylation, which comprises 25 % of the molecular mass, dictates the correct folding and function that are directly associated with the homodimeric quaternary structure [10]. A striking feature in the biosynthetic pathway of PSMA is its trafficking between the ER (endoplasmic reticulum) and the Golgi apparatus and the unusual properties with respect to acquisition of native conformation and intracellular transport [11]. In fact, PSMA exits the ER in an unfolded state at a slow rate and acquires resistance to proteolytic cleavage only after it has reached the medial- or trans-Golgi. Importantly, PSMA does not require complete processing of N- or O-glycans in the Golgi in order to be transported to the cell surface. While several features of the maturation and trafficking pathways of PSMA are resolved, the association of PSMA with the membrane at various stages of its secretory pathway is unknown. These aspects are of primordial importance in the cell physiology of PSMA and towards the assessment of the molecular mechanisms that regulate its expression in prostate cancer.

In the present work we examined the association of PSMA with specialized membranes along the secretory pathway, making use of its extraction properties with a variety of detergents. This is based on the fact that the major constituents of biological membranes – lipids and proteins – determine together the heterogeneous composition of different cell compartments [12] and presumably also of subdomains within the same membrane [13]. It is therefore likely that PSMA would associate with different types of membranes throughout its life cycle, which may regulate its presence in a particular cellular compartment and govern its intracellular transport kinetics. Current concepts have designated these specialized microdomains as ‘lipid rafts’ [14] in virtue of their ability to form a ‘liquid-ordered’ phase that is dispersed into a ‘liquid-disordered’ phase of the lipid bilayer. These microdomains can be biochemically isolated as DRMs (detergent-resistant membranes) upon solubilization with non-ionic detergents such as Triton X-100 [15] and represent tightly packed membrane structures that are enriched in sphingolipids and cholesterol [16]. The biological relevance of DRMs has been demonstrated in numerous cellular events, such as signal transduction pathways [17], cell entry of viral particles [18] and membrane trafficking [19]. Among these concepts, association

Abbreviations used: DPPIV, dipeptidylpeptidase IV; dMM, deoxymannojirimycin; dNM, deoxynojirimycin; DRM, detergent-resistant membrane; endo H, endoglycosidase H; ER, endoplasmic reticulum; mAb, monoclonal antibody; PSMA, prostate-specific membrane antigen; PSMAa, complex glycosylated form of PSMA; PSMAM, mannosereich form of PSMA.

1 These authors contributed equally to this work.

2 To whom correspondence should be addressed (email hassan.naim@tiho-hannover.de).
of membrane proteins with lipid microdomains occurs on the cell surface as well as in intracellular compartments. Several lines of evidence have unequivocally demonstrated that many apical proteins are recruited to Triton X-100-insoluble DRMs in the trans-Golgi network prior to their delivery to the apical membrane [20]. The association of membrane proteins with lipid microdomains or DRMs occurs through the transmembrane domains or glycosylphosphatidylinositol anchors [21] and may implicate accessory proteins which function as receptors for the N- or O-glycans that act as sorting signals [22–25].

Mild detergents other than Triton X-100 have also been utilized in the characterization of membrane microdomains with different lipid and protein compositions [26]. Many membrane proteins that are entirely soluble in Triton X-100 reveal insoluble characteristics with detergents such as Lubrol, Tween 20 or Brij 98 [13,27,28]. Interestingly, the extraction behaviour of the mannose-rich ER-located precursor forms of several apical and basolateral proteins with Tween 20 pointed to the existence of an early pre-Golgi sorting mechanism [29]. It is obvious, therefore, that the heterogeneity of DRMs depends not only on selective enrichment of some lipids and proteins [26], but also on specific intracellular localization and function.

In this paper, we describe how PSMA in human prostate carcinoma LNCaP cells [2] associates with two different types of membrane microdomains. The specific biosynthetic forms of PSMA from distinct intracellular compartments are recruited into lipid microdomains of different composition, thus implicating compartment-specific DRMs in intracellular trafficking.

**MATERIALS AND METHODS**

**Materials**

Tissue culture material was purchased from Greiner Bio-One. RPMI-1640 medium, methionine-free MEM (modified Eagle's medium) and supplemental reagents (penicillin, streptomycin, and glutamine) were from PAA Laboratories. Fetal calf serum, folic acid, trypsin, poly-d-lysine (used to coat tissue culture dishes), Triton X-100, dMM (deoxymannojirimycin), dNM (deoxyxojirimycin), streptavidin horseradish peroxidase, streptavidin agaroase and the proteinase inhibitors pepstatin, leupeptin, aprotinin and trypsin-chymotrypsin inhibitor were from Sigma. PMSF, antipain, and soybean trypsin inhibitor were from Roche. L-[³⁵S]methionine (>1000Ci/mmole), protein A-Sepharose, Hybond-P PVDF membranes and the ECL plus Western blotting detection system were from Amersham Biosciences. Acrylamide, N,N'-methylenebisacrylamide, TEMED, and Tween 20 were purchased from Carl Roth. SDS, ammonium persulfate, and dithiothreitol were obtained from Merck. Endo H (endoglycosidase H) was from New England BioLabs, Brij 58 P from Fluka and Lubrol WX from MP Biomedicals. Sulfoaceticimidobiotin (EZ-Link Sulfo-NHS-Biotin) was from Pierce.

**Antibodies**

Two anti-PSMA mAbs (monoclonal antibodies) were used. The 7E11 mAb [2] binds the cytosolic tail of the protein, whereas the J591 mAb [7] recognizes an extracellular epitope of PSMA. DPPIV (dipeptidylpeptidase IV) from the human colon carcinoma cell line Caco-2 [30] was detected in Western blots by the mAb HBB 3/153 [31]. Commercial mAbs against flotillin-2 and rho A were from Santa Cruz Biotechnology, and the calnexin-specific mAb was from Becton Dickinson. Anti-mouse peroxidase-conjugated secondary antibodies used in Western blotting were from Amersham Biosciences.

**Immunoprecipitation**

PSMA was immunoprecipitated from total lysates of LNCaP cells [2], grown at 60–70% confluence in 10 cm-diameter Petri dishes in RPMI medium supplemented with folic acid (40 mg/l) and 10% fetal calf serum. Cells were biosynthetically labelled with 100 μCi L-[³⁵S]methionine for different time intervals, washed twice with ice-cold PBS and solubilized for 1 h at 4°C in the presence of 1% Triton X-100/PBS and a mixture of proteinase inhibitors (1 mM PMSE, 1 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml antipain and 50 μg/ml trypsin-chymotrypsin inhibitor). After removal of cell debris by centrifugation for 15 min at 9000 g (4°C), PSMA was immunoprecipitated using a combination of the 7E11 and J591 mAbs for 1 h at 4°C. Antigen-antibody complexes were then recovered with protein A-Sepharose, denatured, and loaded on SDS/PAGE (6% gel) under reducing conditions [32]. Proteins were finally detected by autoradiography (using a Phosphor Imager from Bio-Rad) upon exposure to Kodak X-Omat AR films.

In order to obtain an accumulation of newly synthesized proteins in the early compartments, cells were first pulse-labelled with 100 μCi L-[³⁵S]methionine for 30 min at 37°C and then incubated for a further 5 h at 15°C, 20°C or 37°C. Sensitivity of PSMA to trypsin digestion was assayed by incubating immunoprecipitated proteins with 5 μg trypsin for 30 min according to the protocol described previously [11]. In some assays, the sensitivity of PSMA to endo H was also tested after immunoprecipitation [33].

**DRM extraction**

LNCaP cells that reached 60–70% confluence were labelled with [³⁵S]methionine for the time intervals specified in each experiment. After washing twice with ice-cold PBS, cells were solubilized in PBS containing 1% (w/v) detergent (Lubrol WX, Tween 20, Brij 58 P or Triton X-100) and a cocktail of proteinase inhibitors. Cells were first homogenized by trituration with a 21 G needle and then maintained on ice for 1–3 h, depending on the experiment. Afterwards, samples were centrifuged at low speed (15 min at 2000 g) and cell debris was discarded before a centrifugation at 28 300 rev./min (Beckman Type 50.4 Ti rotor), 4°C for 90 min. The supernatant and pellet obtained, corresponding to the soluble and the insoluble fractions respectively, were separately analysed for presence of PSMA. Prior to immunoprecipitation, the pellet fractions were solubilized either under native or denaturing conditions. In order to maintain a native conformation of the proteins, pellets were resuspended in 1 ml 1% Triton X-100/PBS for 1 h. Alternatively, they were first dissolved in 100 μl 1% SDS/PBS, boiled for 5 min at 95°C and then diluted with 900 μl 1% Triton X-100/PBS. After 30 min on ice, the non-solubilized material was removed by centrifugation for 15 min at 9000 g (4°C) and the supernatants were further processed. PSMA from both supernatant and pellet fractions was immunoprecipitated by a combination of the 7E11 and J591 mAbs, which recognize both native and denatured PSMA [11]. When association with lipid microdomains was studied in relation to the glycosylation properties of PSMA, cells were incubated, before and during labelling, with specific inhibitors of N-glycan processing, dNM and dMM, both used at 50 μg/ml.
X-100, Lubrol WX or Tween 20), according to the methodology described above. After the low speed centrifugation step, the sample was diluted to a final sucrose concentration of 40% and layered on to an 80% sucrose cushion. The 1 ml sample was then overlaid with 7 ml of 30% sucrose which was overlaid with 1 ml of 5% sucrose on the top, and finally centrifuged for 18 h at 33,000 rev./min (Beckman SW 40 Ti rotor), 4°C. Nine fractions of 1 ml each were collected from the top and analysed for protein content by Western blotting. The distribution of the marker proteins flotillin-2 and rho A in LNCaP cells was tested as positive and negative control respectively. Confluent Caco-2 cells were used in order to analyse the distribution of human DPPIV and calnexin in similar gradients after solubilization with 0.5% Triton X-100.

**Lipid analysis**

Total lysates of LNCaP cells were prepared as described in the previous paragraph using 1% of Triton X-100, Lubrol WX or Tween 20, all dissolved in PBS. Pellets obtained after 90 min of centrifugation at 28,300 rev./min (Beckman Type 50.4 Ti rotor), 4°C were washed twice with chilled PBS (at each step, samples were centrifuged for 10 min at 15,000 g, 4°C). Phospholipids, sphingolipids and cholesterol were extracted from the pellets following the method of Bligh and Dyer [35]. The content in different classes of phospholipids was further analysed according to Meyer zu Dutttingdorf et al. [36]. Results are represented as mean values ± S.D., based on four independent experiments.

**DRMs at the cell surface**

Cell surface proteins in LNCaP cells were biotinylated according to the protocol described in [11]. Following solubilization with 1% Lubrol WX/PBS, the samples were centrifuged at 40,100 rev./min (Beckman Type 100 Ti rotor), 4°C, to recover the Lubrol WX-insoluble membranes, as described above. In a control experiment, biotinylated cells were lysed with 1% Triton X-100/PBS instead. PSMA was immunoinosolated from the total lysate, as well as from the supernatant and pellet fractions, and equally divided into two parts to be analysed by SDS/PAGE and Western blotting. The use of streptavidin allowed the selective detection of PSMA on the cell surface, whereas PSMA-specific antibodies were used to monitor the total amount of PSMA as comparison. As a supplemental control, biotinylated proteins were precipitated first using streptavidin-agarose beads and analysed by Western blotting using PSMA antibodies instead.

PSMA was also immunoprecipitated from the cell surface of radiolabelled LNCaP cells using the J591 mAb dissolved in 3 ml of chilled RPMI medium [11]. After 45 min on ice in the presence of the anti-PSMA antibody, the cells were solubilized for 1 h with 1% Lubrol WX/PBS together with lysate from unlabelled LNCaP cells as a source of competing PSMA molecules for the unbound antibodies. The soluble and insoluble materials were separated by ultracentrifugation (see above) and PSMA was recovered by addition of protein A-Sepharose. The pellet fractions were resuspended in 1% Triton X-100/PBS to preserve the antigen-antibody interactions. In addition, intracellular PSMA from both soluble and insoluble fractions was immunoprecipitated with the combination of mAbs 7E11 and J591. Proteins were finally separated by SDS/PAGE (6% gel) and analysed by autoradiography.

**Analysis of the quaternary structure**

Monomers and dimers of PSMA molecules from LNCaP cells were separated by sucrose-density gradients under native conditions and then immunoinosolated. Cells were pulse-labelled for different time intervals with 100 µCi L-[35S]methionine, washed and lysed for 2 h with 6 mM dodecyl-β-maltoside dissolved in 100 mM NaCl and 50 mM Tris/HCl, pH 7.5 [37]. After a centrifugation step at 10,000 g to remove cell debris, cell lysates were loaded on to the top of 5–25% (w/v) continuous sucrose gradients which were centrifuged at 23,700 rev./min (Beckman SW 40 Ti rotor) for 18 h (4°C). Fractions of 500 µl each were collected and immunoprecipitated for the detection of PSMA (see above). As a control, distribution of human DPPIV was also investigated by performing a gradient under the same conditions from a lysate of Caco-2 cells pulse-labelled with L-[35S]methionine for 30 min.

**RESULTS**

**Biosynthesis of PSMA in LNCaP cells**

PSMA is endogenously overexpressed in LNCaP cells, a line derived from a human prostate adenocarcinoma which had metastasized to the lymph nodes [11]. One initial aim of the present study was to determine the biosynthetic features and intracellular transport of newly synthesized PSMA molecules in this cellular model. PSMA was immunoisolated using a combination of two mAbs, recognizing the luminal tail of the protein (J591) and an intracellular epitope downstream of the transmembrane domain (7E11). The two main biosynthetic forms of PSMA in LNCaP cells are the PSMA_\text{M} (mannose-rich form) and the PSMA_\text{C} (complex glycosylated form), as assessed by their reactivity towards endo H [33] (Figure 1A). In LNCaP cells, the PSMA_\text{C} is only slightly larger than the PSMA_\text{M}, and differs markedly in this respect from that generated in transfected COS-1 cells [11] thus pointing to variations in the extent of glycan processing in the Golgi compartment of these two cell types. Nevertheless, the conversion rate of the precursor protein into the complex PSMA is conserved (results not shown). The conversion of PSMA to a protease-resistant correct conformation occurs in LNCaP cells at a later stage of biosynthesis. In fact, the ER-resident form, PSMA_\text{H}, was completely sensitive to trypsin, whereas the different classes of phospholipids was further analysed according to Meyer zu Dutttingdorf et al. [36]. Results are represented as mean values ± S.D., based on four independent experiments.

**Figure 1 PSMA acquires resistance to trypsin as a complex glycosylated protein**

(A) PSMA was immunoprecipitated from total lysates of sub-confluent LNCaP cells that were pulse-labelled for 30 or 180 min with [35S]methionine. Digestion with endo H enzyme allowed discrimination between PSMA_M (upper bands) and PSMA_C (lower bands). (B) After 30 min of labelling at 37°C, LNCaP cells were maintained for a further 5 h at the indicated temperatures (15, 20 or 37°C). Following lysis and immunoprecipitation with the 7E11 and J591 mAbs, PSMA was left untreated (−) or treated with 5 µg trypsin (+). Proteins were finally subjected to SDS/PAGE on a 6% acrylamide gel and analysed by autoradiography. © The Authors Journal compilation © 2008 Biochemical Society
protein became trypsin resistant after acquisition of complex glycosylation (Figure 1B). These findings were corroborated in a series of temperature block experiments combined with protease treatments. LNCaP cells that were metabolically labelled with L-[35S]methionine at 37°C and subsequently chased at 15°C possessed only trypsin-sensitive PSMAM, due to the transport block of the protein in the early secretory pathway. At 20°C, PSMA reaches the Golgi and is blocked there. Processing of PSMA at this temperature to a complex form is not efficient and the majority of the protein is retained in the mannose-rich glycosylated form that maintained a trypsin-sensitive conformation. Further incubation of the cells at 37°C allowed formation of mature PSMA that acquires trypsin-resistance and also presumably acquires a folded structure. Similarly, when cells were labelled continuously at 37°C, both PSMAM and PSMAC were obtained, but only the mature form was trypsin-resistant. These results are comparable with previous findings in COS-1 cells [11] and unequivocally indicate that, regardless of the cellular system, the folding process of PSMA extends from the ER to the Golgi, the site of complex glycosylation, and therefore represents a unique property of the PSMA protein.

Association of PSMA with DRMs of different composition

Many transmembrane proteins associate with lipid components in the membrane bilayer, forming structural and functional platforms or microdomains. These platforms can play a crucial role in fundamental cellular events, including intracellular trafficking and protein sorting in polarized epithelial cells [20,23]. Experimentally, membrane microdomains can be isolated based on their insolubility in non-ionic detergents [15]. Upon solubilization of cellular extracts with a detergent under controlled conditions, the detergent-resistant membranes can be recovered either by sedimentation or in the floating fractions of sucrose-density gradients. We first set out to investigate whether PSMA associates with sphingolipid/cholesterol-enriched Triton X-100-insoluble DRMs, which have been initially characterized to be implicated in protein transport and sorting [21]. In a first set of experiments, we analysed the solubility properties of PSMA in LNCaP cells that were labelled for 5 h with [35S]methionine, during which time the mannose-rich and complex glycosylated biosynthetic forms could be identified. Figure 2(A) shows that both PSMAM and PSMAC were completely extractable with 1% Triton X-100. Recent observations have demonstrated the existence of novel types of DRMs with structures that differ in their lipid composition from the Triton X-100 DRMs and contain proteins that are usually entirely soluble in Triton X-100 [29]. These DRMs can be extracted with several non-ionic detergents, such as Lubrol WX, Tween 20 and Brij 58. We asked therefore whether (i) DRMs containing PSMA do exist and (ii) if these DRMs could discriminate between the two major biosynthetic forms of PSMA. As shown in Figure 2(B), PSMA was found to be partially insoluble in Lubrol WX, Tween 20 and Brij 58. More importantly, PSMAC and PSMAM were differentially partitioned into the soluble and insoluble DRM-containing fractions. In particular, the majority of PSMA molecules insoluble in Tween 20 were of the mannose-rich form. Relative amounts of PSMAC and PSMAM in each fraction are graphically represented in Figure 2(C). The two other detergents, Lubrol WX and Brij 58, solubilized PSMA in a comparable manner as assessed by the similar patterns of protein distribution (Figure 2C). In fact, after 5 h of labelling, the ratio of PSMAC in the insoluble pellet fraction of both detergents amounted to three times as much as in the Tween 20 pellets, whereas the soluble fraction mostly contained the mannose-rich form.

The detergent extractability of PSMA was also evaluated by sucrose-density centrifugation, based on the ability of DRM structures to float and be recovered on the top of sucrose gradients [34]. Due to the similar distribution profile of PSMA in the presence of Lubrol WX and Brij 58, we focused on Lubrol WX and compared the extraction profiles with those of Tween 20. As shown in Figure 3(A), PSMA was not detected in the floating fractions of the discontinuous sucrose gradient when Triton X-100 was utilized. As a control, we used a membrane glycoprotein that is associated with Triton X-100-specific DRMs, the intestinal DPPIV expressed in Caco-2 cells (Figure 3B). DPPIV was recovered in the floating fractions upon solubilization with Triton X-100, confirming previous results on the association of this protein with Triton X-100-insoluble DRMs [24]. In the same gradient, we examined the distribution of calnexin, a membrane-bound chaperone of the ER, and showed that this protein was not retained in the DRM fractions [38]. These results demonstrate unequivocally that PSMA is not associated with Triton X-100-insoluble DRMs. Furthermore, we analysed the distribution of PSMA in sucrose gradients of cellular extracts obtained with Tween 20 and Lubrol WX. As shown in Figure 3(A), the upper
Interaction of PSMA with DRMs

Figure 3 Isolation of DRMs by sucrose-density gradient centrifugation

(A) Lysates of LNCaP cells obtained after solubilization with 0.5% Triton X-100 (TX-100), Tween 20 or Lubrol WX were subjected to a discontinuous sucrose-density gradient (1 ml 5% / 7 ml 30%). After centrifugation, one third of each fraction was concentrated and analysed by SDS/PAGE (6% gel) and Western blotting for the presence of PSMA. (B) For Triton X-100, the distribution profiles of DPPIV and calnexin from Caco-2 cells were considered as positive and negative controls respectively. (C) For all three detergents, the distribution of PSMA in LNCaP cells was compared with flotillin-2 and rho A, taken as positive and negative controls regarding DRM association. Lys, total lysate prior to fractionation by sucrose gradient.

fractions of the sucrose-gradients contained PSMA when Tween 20 and Lubrol WX were used, thus supporting the results obtained in the ultracentrifugation experiments (Figure 2). As positive and negative controls for all three detergents, we used two protein markers that are DRM-associated and non-associated respectively. As shown in Figure 3(C), flotillin-2 was recovered in the DRM floating fractions, while rho A was retained in the bottom fractions of the gradients, thus supporting the finding that PSMA is associated with Tween 20- and Lubrol WX-DRMs.

Lipid composition of membranes isolated with different detergents

In view of the differential association of PSMA with DRMs corresponding to cellular extraction with Lubrol WX and Tween 20 but not Triton X-100, we performed a comparative analysis of the lipid contents of the DRMs. Figure 4(A) shows a substantial decrease in the amount of sphingolipids and cholesterol upon solubilization with Lubrol WX and Tween 20 as compared with Triton X-100, where DRMs are enriched in these two lipids [21]. The relative proportions of different classes of phospholipids have been resolved by HPLC and are depicted in Figure 4(B). The most remarkable difference is the proportion of phosphatidylethanolamine, which is increased approx. 6- and 8-fold in the DRMs of Tween 20 and Lubrol WX respectively. Quantitative differences emerged also by comparing membranes isolated with Lubrol WX and Tween 20. In particular, the proportion of phosphatidylinositol, phosphatidylserine and phosphatidylcholine differed significantly, suggestive of specific physicochemical properties discriminating the two distinct types of DRMs.

Association with DRMs during biogenesis of PSMA

The differential association of the biosynthetic forms of PSMA, i.e. PSMA_M and PSMA_C, with Tween 20- and Lubrol WX-DRMs (Figure 2), as well as the differences in the lipid composition of these DRMs, raised the question of a possible crucial role of these entities as platforms that dictate the various phases of PSMA trafficking. To this end, LNCaP cells were subjected to pulse–chase with [35S]methionine and lysed with either Tween 20 (Figures 5A and 5B) or Lubrol WX (Figure 5C). DRMs were then isolated by sedimentation, followed by immunoisolation of PSMA. After 30 min of pulse, the protein was still completely in its mannose-rich form, with an ER localization. Interestingly, the use of Tween 20 allowed the discrimination between two distinct forms of PSMA (Figure 5A). As shown in Figure 5(B), flotillin-2 was recovered in the DRM floating fractions, while rho A was retained in the bottom fractions of the gradients, thus supporting the finding that PSMA is associated with Tween 20- and Lubrol WX-DRMs.

Figure 4 Lipid composition of DRMs in LNCaP cells

DRMs were extracted from LNCaP cells using 1% Triton X-100, Lubrol WX or Tween 20 and recovered by sedimentation. (A) The lipid content is presented as relative proportion of either cholesterol (Chol) or sphingolipids (SL) with respect to total phospholipids (PL). (B) The phospholipids were further resolved by HPLC into the following classes (each reported as a percentage of total phospholipid): phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM). Results are given as mean ± S.D. based on four independent experiments.
soluble in TWEEN 20. This clearly indicates that the localization of TWEEN 20-insoluble DRMs containing PSMA is on the early secretory pathway. The use of Lubrol WX gave a completely different pattern (Figure 5C). Both ER forms of PSMA...form appeared as a very faint band at the 30 min chase point, also in the soluble fraction. Subsequently, PSMA...in association with Lubrol WX-insoluble DRMs as well (60 min chase). Recovery of PSMA...in the DRM or pellet fraction increased and reached a maximum after 120 min of pulse–chase.

In view of the association of one glycoform of PSMA with TWEEN 20-DRMs, we determined whether this association at the early stages of the secretory pathway depends on the glycosylation state of the protein. To this end, the glycoforms were regulated using dNM, an inhibitor of the ER-located glucosidase I, which is responsible for the initial removal of the terminal glucose of the core glycan chain in the ER [39]. Treatment of LNCaP cells with dNM during a labelling period of 30 min resulted in the detection of a slightly higher band that was recovered in the DRM and also in the soluble fraction upon solubilization with TWEEN 20 (Figure 6A). It is therefore likely that the subcellular localization rather than the glycan moiety determines the association of PSMA with DRMs. To substantiate these results, we examined the influence of modification of the sugar chains in the Golgi compartment on the association of PSMA with Lubrol WX-DRMs. For this we utilized dMM, a modulator of complex glycosylation through inhibition of Golgi mannosidase I [39]. As shown in Figure 6(B), PSMA generated in the presence of dMM revealed an apparent molecular mass similar to that of the mannos-rich polypeptide and is also endo H-sensitive (results not shown). Despite this, this form was associated with Lubrol WX-DRMs as its complex glycosylated PSMA counterpart obtained in the absence of dMM. In conclusion, the subcellular location of PSMA dictates its association with TWEEN 20- or Lubrol WX-DRMs.
Interaction of PSMA with DRMs

**Figure 7** PSMA associates with Lubrol WX-insoluble DRMs on the cell surface

(A) PSMA was selectively isolated from the cell surface of LNCaP cells pulse-labelled for 5 h. The Jo591 mAb which recognizes an extracellular epitope of the protein was used. Cells were then lysed for 1 h with 1% Lubrol WX followed by centrifugation at 40 100 rev./min. (Beckman Type 100 Ti rotor) for 1.5 h to obtain a soluble (S) and an insoluble (P) fraction. After recovery of cell surface PSMA (PM) by addition of protein A-Sepharose, intracellular PSMA was immunoprecipitated from the remaining lysate (I). For comparison, surface-immunoprecipitation was also performed on LNCaP cells lysed by Triton X-100 for the total amount of PSMA (Total). (B) LNCaP cells were cell-surface biotinylated and lysed with 1% Lubrol WX. After centrifugation the insoluble fraction (P) was solubilized with 1% Triton X-100 and PSMA was immunoprecipitated from both insoluble (P) and soluble (S) fractions. Here, the amount of PSMA detected by streptavidin (PM) is compared with the total amount of PSMA in insoluble and soluble fractions, based on the use of anti-PSMA mAbs in Western blotting. (C) As a supplemental control for the amount of surface PSMA found in Lubrol rafts, biotinylated proteins were precipitated using streptavidin beads. Western blot analysis of these proteins for the presence of PSMA resulted in a ratio similar to that in (B). Tot, LNCaP cells lysed by Triton X-100 for the total amount of PSMA; P, supernatant of the streptavidin bead immunoprecipitate of the insoluble fraction; S, supernatant of the streptavidin bead immunoprecipitate of the soluble fraction.

**Figure 8** PSMA dimerizes within a short time of acquiring its complex glycosylated form

LNCaP cells were pulse-labelled with [35S]methionine for 15 or 45 min (P, upper two panels). Alternatively, they were pulsed for 45 min and then chased (C) for a further 4 h to follow the fate of the pulse-labelled PSMA molecules. Cells were solubilized with 6 mM dodecyl-β-maltoside and 1 ml of the lysate was loaded on to the top of a 5–25% continuous sucrose gradient. After 18 h of centrifugation at 23 700 rev./min (Beckman SW 40 Ti rotor), PSMA was immunoprecipitated from each fraction. The distribution of DPPIV was tested under the same conditions following labelling of Caco-2 cells for 30 min. Shown are fractions 4–16 taken of twenty 500 µl-fractions collected in each experiment.

**Discussion**

PSMA is a potential target for prostate cancer treatment [5–7], as well as for solid tumours of different histotype due to its de novo expression in the tumour neovasculature [4]. Several aspects of its structure, biosynthesis and processing have been elucidated, some of which have revealed surprising findings. In fact, PSMA exits the ER in an unfolded, trypsin-sensitive form and acquires a correctly folded native conformation in the Golgi compartment [11]. This folding pattern cannot be ascribed to an impairment of the protein synthesis machinery in heterologous systems [11], since we observed a similar maturation pattern in the present study in LNCaP cells, which express the protein endogenously [2]. This maturation behaviour does not entirely conform to current concepts of protein folding and quality control mechanisms in the ER. As such, PSMA is a unique example of a membrane protein and unravelling of the molecular mechanisms underlying its trafficking and targeting may lead to the identification of novel pathways or a modification of established concepts. From a mechanistic point of view it is important to understand the mode of association of PSMA with membranes on its way to the cell surface. This is an important task given our current knowledge which has assigned a role for membrane microdomains as platforms in prostate carcinoma which may influence cancer aggressiveness in malignant transformation [41,42]. Two distinct caveolin-independent but cholesterol-dependent signalling pathways have been identified in LNCaP cells [43,44].

Possible interactions of PSMA with membranes in LNCaP cells had not been analysed previously. We therefore set out to investigate the possible association of PSMA with lipid microdomains at different biosynthetic stages. Extraction with distinct detergents allows isolation of DRMs with a different composition [26], which can play a physiological role not only at the cell surface [13] but also in the trans-Golgi network and in earlier compartments, where they are involved in cellular trafficking of transmembrane proteins [29]. Our results support the existence of a functional as well as spatial correlation between trafficking of PSMA and the lipid content of DRMs.

First, PSMA does not associate with Triton X-100-resistant DRMs that are enriched in cholesterol and sphingolipids [21].
Association of transmembrane proteins with this class of membrane microdomains represents a mechanism of apical protein sorting occurring at the level of the trans-Golgi network in polarized epithelial cells [20]. Although PSMA is localized at the apical membrane [9,11] and shares striking homology with the apical protein DPPIV [45] that is associated with cholesterol- and sphingolipid-rich DRMs [24], PSMA is entirely soluble in Triton X-100. Nevertheless, the extraction properties of PSMA in Tween 20 and Lubrol WX suggested a crucial role of the DRMs in the trafficking of PSMA. In fact, the two major glycoforms of PSMA reveal distinct extractability profiles with these detergents. Thus, the mannose-rich form associates predominantly with Tween 20-DRMs and the complex glycosylated form is mainly insoluble in Lubrol WX, consistent with the view that PSMA is transported via different microdomains along the secretory pathway (Supplemental Figure 2, at http://www.BiochemJ.org/bj/409/bj4090149add.htm). These various DRMs could also be distinguished on the basis of their distinct lipid contents that differ substantially from the composition of Triton X-100 DRMs. Even though DRM extraction with detergents per se is not informative about the spatial localization of membranes, the different biosynthetic forms of PSMA can be considered as a marker for cellular distribution. The selective partitioning of PSMAM and PSMAC in distinct DRMs is indicative of an enrichment of Tween 20-DRMs containing PSMA in the ER and of Lubrol WX-DRMs in later compartments.

There is growing evidence supporting diverse modes of association of transmembrane proteins with microdomains along the secretory pathway. Association with Tween 20-insoluble DRMs in the ER, for instance, has been described as an early sorting mechanism in polarized cells [29]. Protein association with lipid microdomains in the early secretory pathway has also recently been reported for ER-resident proteins [46]. Detergents other than Triton X-100 have already been used to isolate membrane structures from the trans-Golgi network as well as from the cell surface. This is the case for the complex glycosylated protein prominin [13], which associates on the cell surface of epithelial cells with DRMs that are soluble in Triton X-100 but insoluble with Lubrol WX, i.e. in a similar way to PSMA in LNCaP cells. These findings also imply the coexistence of distinct lipid microdomains within the same cell compartment. In fact, whereas several lines of evidence demonstrated the implication of Triton X-100 DRMs in processes taking place at the plasma membrane [17,18], the isolation of PSMA-containing DRMs showed the existence of at least one other type of protein/lipid platform of different composition at the cell surface. Moreover, it is the first time that a switch between membrane structures has been described along the maturation pathway of a protein. After interacting with Tween 20-insoluble membranes in the ER, PSMA associates with Lubrol WX-DRMs as soon as it enters the Golgi, where it acquires complex glycosylation. The interaction with Lubrol WX-DRMs is either maintained or renewed once it reaches the plasma membrane. At the steady state, a major part of the PSMA molecules are present at the cell surface and most of them associate with Lubrol WX-DRMs. Since DRMs are dynamic structures in the membranes, the soluble PSMA and the DRM-associated form of the protein are most likely in equilibrium with each other. Whether the existence of Lubrol-DRMs on the cell surface has a physiological function remains to be elucidated.

Our results suggest that the conversion of PSMA into a complex glycosylated protein is a process spatially related to association with Lubrol WX-insoluble membranes. Also related to association with DRMs is the homodimerization of PSMA, which occurs within a short time of accomplishing complex glycosylation, which is also taking place in the Golgi. This is supported by the observation that PSMA is found as a dimeric protein, whereas the ER forms are exclusively present as monomers. It has been shown that dimerization of PSMA and acquisition of a functional structure are strictly related to each other [10]. While it is clear that dimeric PSMA associates with Lubrol WX-insoluble membranes, it is not obvious whether the association with DRMs precedes or follows the dimerization.

In conclusion, the present investigation demonstrates a novel pathway of intracellular protein transport that is dictated by the association of a glycoprotein with distinct membrane microdomains at different stages of its maturation. The identification of DRM-associated PSMA at the plasma membrane of LNCaP cells might be an important step towards unravelling the transition of prostate cells to a neoplastic phenotype and the potential role of these DRMs as signalling platforms at the cell surface. The outcome of these studies is equally important in designing strategies of immunotherapy comprising PSMA-expressing cells.