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Human platelet P-235, a talin-like actin binding protein, binds selectively to mixed lipid bilayers

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The interaction of platelet talin (P-235) with mixtures of dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG) and dimyristoylphosphatidylserine (DMPS) as well as with pure lipids was studied in reconstituted lipid bilayers. Incorporation of platelet talin into vesicles was achieved by self-assembly during cycles of freeze-thawing of co-dispersions containing vesicles and the purified protein. The yield of protein incorporation as a function of lipid composition was determined by measuring the protein/lipid ratio using protein assay, phosphate determination and gel electrophoresis in parallel. Protein–lipid interactions are monitored by high sensitive differential scanning calorimetry (DSC) measuring (i) the shifts of transition states ΔT_s^* and ΔT_l^*, where T_s represents the solidus line, the onset of lipid chain melting, and T_l the liquidus line, the endpoint of chain melting, and (ii) the heats of transition. Cytoplasmic talin differs from a membrane bound form by its ability and mode of lipid interaction. The latter partially penetrates into the hydrophobic region of the bilayer, which renders a low incorporation rate even into neutral lipids. This interaction is greatly enhanced in the presence of charged lipids: a marked shift of T_l occurs due to a selective electrostatic interaction of the protein with the membrane surface. Evidence for a selective binding is also provided by Fourier transform infrared spectroscopy (FTIR). Right-side-out oriented platelet talin can be cleaved by proteinases, which truncate the extrinsic electrostatic binding domain but not the hydrophobic. In addition, reconstituted platelet talin, like in vivo, can be cleaved by thrombin. The interaction of cytoplasmic platelet talin with lipid bilayers is purely electrostatic. Our data suggest that protein reconstitution by freeze-thawing is an equilibrium process and that the protein distribution between the membrane and water is determined by the Nernst distribution law. Consequently, the work of protein transfer from water into the bilayer can be measured as a function of charged lipids.

Introduction

Talin, a 235 kDa protein is believed to be involved in generating a transmembrane linkage between the cytoskeleton and extracellular matrix proteins [1]. It was first recognized to be part of adhesion plaques in fibroblasts [2] and was later shown to bind to the fibronectin receptor [3]. On the cytoplasmic site, talin could bind at least to vinculin [4]. Since vinculin was identified as an actin-binding protein [5,6] and the relevant epitope for actin binding could be identified [7], a transmembrane linkage on the protein level could basically exist.

In platelets, a talin-like protein was isolated and named P-235 [8]. During platelet activation P-235 (platelet talin) is cleaved into 46 kDa and 190–200 kDa subunits by a Ca^{2+}-dependent endogenous proteinase, calpain II. In subsequent studies Beckerle and collaborators showed that platelet P-235 and smooth muscle talin are closely related [9] and that it is indeed talin which is implicated in platelet activation [10]. Even more interestingly, platelet talin during activation was shown to be redistributed from the cytoplasm towards the plasma membrane [11,12].

Since the organization of talin-containing adhesion foci within the membrane can obviously occur without binding to accessory proteins, e.g., vinculin [13], it is tempting to investigate the interaction of talin with lipid membranes per se. In particular, we have concentrated on the role of charged lipids for the associa-

Abbreviations: DSC, differential scanning calorimetry; FTIR, Fourier transform infrared spectroscopy; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DMPS, dimyristoylphosphatidylserine; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethyleneglycol bis(2-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; DTT, dithiothreitol, SDS, sodium dodecyl sulfate.

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tion of platelet talin with vesicles. The reconstitution of protein containing vesicle membranes (proteo-vesicles) was achieved by cyclic freeze-thawing of a suspension of sonicated vesicles and solubilized protein. The interaction of the protein with lipids is quantified in terms of shifts of phase boundaries of lipid mixtures caused after protein incorporation using high sensitivity differential scanning calorimetry. Further information on the selectivity of lipid-protein interactions is obtained by FTIR-spectroscopy after partial deuteration of one lipid component. The right-side-out incorporation of the protein within proteo-vesicles is verified by proteinase cleavage of protein head groups. The applied methods will allow further studies of more complex interactions between cytoskeletal proteins and membranes.

Materials and Methods

Materials

DMPC and DMPG were commercial products from Sigma and Avanti Polar Lipids. DMPS was a gift of Dr. G. Cevc, Technical University of Munich. Triton X-100 was purchased from Serva Feinbiochemicals and metrizamide from Fluka. Buffer reagents were purchased from Sigma. Tris-buffer C: 10 mM Tris-HCl, 3 mM EDTA, 1 mM EGTA, 0.1 mM DTT (pH 8.0).

Methods

P-235 (platelet talin) purification. The protein was isolated from outdated platelet concentrates (6–14 days old) obtained from the Bayerisches Rotes Kreuz (BRK) and purified by the procedure of Collier and Wang [8] with the following modifications: (1) EGTA (1 mM) was added to all buffers to prevent calcium-dependent proteolysis. (2) PMSF (1 mM) was included in the extraction buffers to inhibit proteinase activation. (3) Platelets were first lysed by detergent-free buffer to extract the water soluble proteins. After centrifugation for 1 h at $75000 \times g$ the remaining pellet was reextracted with Triton-X-100 containing extraction buffer. (4) The sepharose Cl-6B column was substituted for the Bio-Gel A-5m column.

Reconstitution into lipid vesicles. 2 mg of lipid were dissolved in organic solvents and dried by evaporation under nitrogen. Chloroform was used for DMPC and DMPC/DMPG mixtures and chloroform/methanol (3:1) for DMPC/DMPS mixtures. Residual solvent was removed by storage in an excicator for 12 h. The lipid film was reswollen by adding 1 ml Tris-buffer C and sonicated for 5 min with a Branson Sonifier (B15). To avoid overheating, the solution was kept in a cooling bath of 30 °C. The average size of the vesicles was about 100 nm. The protein dissolved in Tris-buffer C was added in varying concentrations (see below). The samples were shock-frozen in liquid nitrogen and reheated to room temperature up to five times. Before DSC measurements the samples were kept at 30 °C for 15 min to restabilize. For the subsequent DSC-measurements the volume of all vesicle suspensions was adjusted to 1.7 ml with Tris-buffer C.

Density gradient centrifugation. The separation of protein free and protein loaded vesicles by density gradient centrifugation was an essential procedure to determine the percent of protein incorporated into the vesicles. The density gradient column was made up of three layers: the first containing 0.64 g metrizamide in 1.6 ml of the initial vesicle preparation in Tris-buffer C. Upon this a solution of 0.3 g metrizamide in 1.5 ml Tris-buffer C was added which was finally covered with 700 µl water. After centrifugation of the samples for 2 h at $100000 \times g$, vesicles appeared in the upper part of the column, while the non-bound protein was concentrated at the bottom of the gradient (Fig. 3). Small aliquots were taken from the vesicle and the bottom fraction in order to determine the percentage of protein incorporated into the vesicles by protein and phosphate determination.

Protein determination was carried out according to Lowry et al. [14] as modified by Peterson [15].

Phosphate determination. Phosphate was determined as described by Ammon and Hinsberg [16] to calculate the mass fractions of protein and lipid in reconstituted vesicles. Briefly, vesicle samples were taken from individual bands (V_I and V_{II}, Fig. 3c) after density gradient centrifugation and dried in glass flasks which were washed in phosphate free solvent and bidistilled water previously. The dried samples were dissolved in 140 µl of 72% perchloric acid and ashed by heating at 200 °C for 90 min. After cooling, 700 µl of a solution made up of 125 ml ammonium molybdate, 125 ml perchloric acid and 450 ml bidistilled water was added to form a phosphomolybdate complex which was then reduced with ascorbic acid (3%, w/w). The mixture was heated in boiling water for 5 min. After cooling the absorbance was measured at 810 nm. The absolute mass of lipid could be determined by comparison with a standard calibration solution of Na_2HPO_4.

Calorimetry. DSC measurements were performed with a high sensitivity MC-2 microcalorimeter (Microcal, Amherst, MA, U.S.A.). The data were stored and analysed by an IBM AT computer using the DA-2 software provided by Microcal. Vesicles with and without reconstituted platelet talin were precooled on ice and then filled into the calorimeter. The heating scan was started after an equilibration time of 20–30 min at 5 °C, the starting temperature of the hs-DSC scan, with a scan rate of 90 C°/h and a 15 s time increment (filter constant) between each data acquisition. The transition enthalpies, ΔH, were determined after subtraction of the buffer baseline.

FTIR-experiments. Infrared spectra of the vesicle suspension were taken with a Nicolet 60 S X B FTIR spectrometer equipped with a liquid ATR accessory (thermostated 5 ml cell with ZnS crystal from Spectratech) and a MCT detector. The temperature was measured at the sample cell using a Pt 100 thermocouple which controlled an external water bath and was constant within ±0.2 C°. Measurements were performed from lower to higher temperatures for each sample. At each temperature 100 scans were accumulated at a resolution of 2 cm^{-1} between 4000 and 400 cm^{-1}. Buffer spectra were recorded separately at identical temperatures and interactively subtracted from the sample spectra. The frequency of the methylene stretching vibrations was determined at the maximum of the corresponding bands.

Results

Assay conditions

To test whether the purified protein fraction of 230–240 kDa (Fig. 1a) is indeed platelet talin, 100 μg of the purified protein were incubated at 37°C with 1 unit thrombin. As is clearly seen in Fig. 1b, platelet talin is cleaved into 200 kDa and 46 kDa subunits.

Calorimetric studies of the interaction of platelet

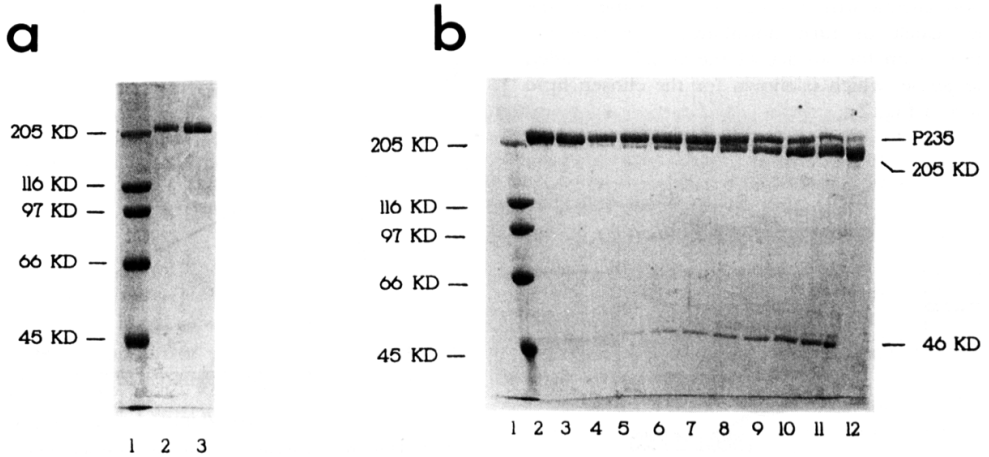

Fig. 1. (a) Silver-stained SDS-gels (7.5%) of purified platelet talin extracted with a Triton-X-100 containing extraction buffer. Protein standards lane 1; 1 and 7 μg purified protein, lane 2 and 3. (b) Cleavage of platelet talin by thrombin. Coomassie-blue stained SDS-gels (7.5%). From left to right: protein standard, lane 1; control sample of purified platelet talin without thrombin treatment, lane 2; cleavage by thrombin with rising incubation time 0–130 min, lane 3–12.

Fig. 2. Freeze-fracture electron micrograph of a vesicle composed of DMPC and DMPG (1:1) showing the typical 'ripple phase' ($P_{\beta'}$) on the vesicle surface (freeze-fracture temperature: 17°C. Magnification × 46000.

talin with model membranes are presented in Figs. 4–11. The specific heat of the vesicle suspensions is recorded as a function of temperature between 10 °C and 35 °C in order to observe the effects of the protein on the main (L_α–$P_{\beta'}$) and the pretransition ($L_\beta - P_{\beta'}$). With DMPC, DMPG and DMPS the peak of the main transition is centered at 23 °C and corresponds to the transition from the low-temperature crystalline phase $P_{\beta'}$ to the liquid temperature phase L_α and describes the chain melting behaviour. The peak of the pretransitions centered at 14–15 °C is caused by the transition from high- ($P_{\beta'}$) to low-temperature crystalline (L_β) modification. The $P_{\beta'}$ phase is distinguished by the tilt of the hydrocarbon chains with respect to the plane of the membrane which in turn undulates. The resulting banded pattern on the surface of the vesicles is called the 'ripple phase' which is shown for the chosen lipid composition in Fig. 2.

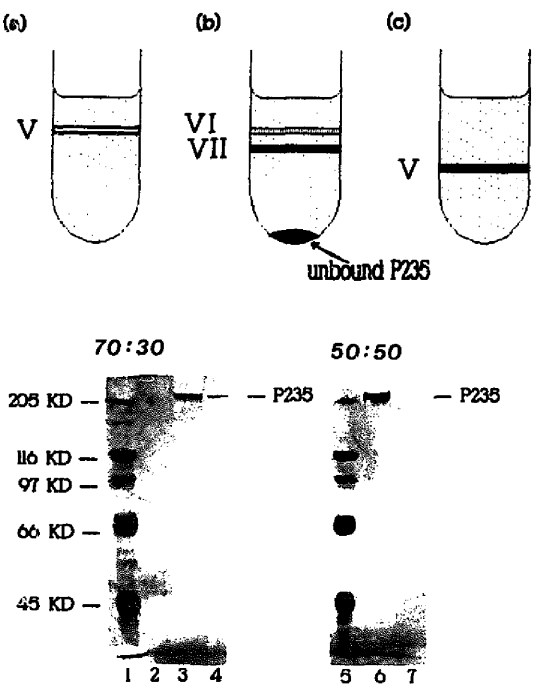

Fig. 3. Distribution of protein and vesicles after reconstitution of platelet talin into vesicles and subsequent density gradient centrifugation. Schematic view of density gradient columns: (a) DMPC/DMPG mixtures without protein (control), (b) DMPC/DMPG (70:30) mixtures. (c) DMPC/DMPG (50:50) mixtures. Corresponding SDS-gels show reconstituted vesicles in a DMPC/DMPG ratio of 70:30 (lane 2–4) with the unloaded vesicle fraction V_I, lane 2, the reconstituted vesicle fraction V_{II}, lane 3 and the unbound protein containing bottom fraction, lane 4. Reconstituted vesicles in a DMPC/DMPG ratio of 50:50 are shown in lanes 5–7, with the vesicle fraction, lane 6, and the bottom fraction, lane 7. Lanes 1 and 5 show protein standards.

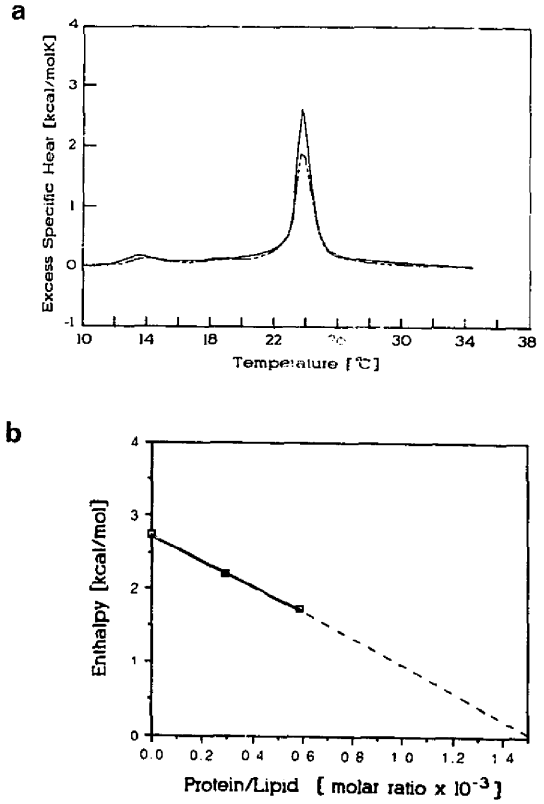

Fig. 4. DSC-measurements of pure and platelet talin-reconstituted DMPC vesicles. (a) Temperature dependence of specific heat (in kcal/mol) of pure DMPC vesicles (———) and platelet talin containing DMPC vesicles (- - - - -) prepared by the freeze-thaw technique. The initial protein/lipid molar ratio was 1:1700. (b) Variation of the heat of transition, ΔH, with increasing protein content.

DMPC vesicles

In Fig. 4 the specific heat vs. temperature plots are shown for pure DMPC vesicles and for proteo-vesicles prepared by the freeze-thaw technique. Density gradient centrifugation showed the existence of two vesicle fractions in these preparations: a protein free band (V_I) and a protein loaded band (V_{II}), Fig. 3b. The bands were separated and a phosphate determination showed that the mass ratio of the two populations was about 1:1. Measurements of protein contents in band V_{II} and the bottom fraction showed that 37% of the total protein was incorporated into the vesicles when the initial protein/lipid molar ratio was $x^0_{P/l} = 2.9 \cdot 10^{-4}$ (1:3500), Table I. Indeed, in the thermograms of Fig. 4 the positions and the widths of the main and the pretransition of DMPC are hardly influenced. However, as shown in Fig. 4b the heat of the L_α–$P_{\beta'}$ transition, ΔH, decreases linearly with increasing protein concentrations. This indicates that there is a weak but stable

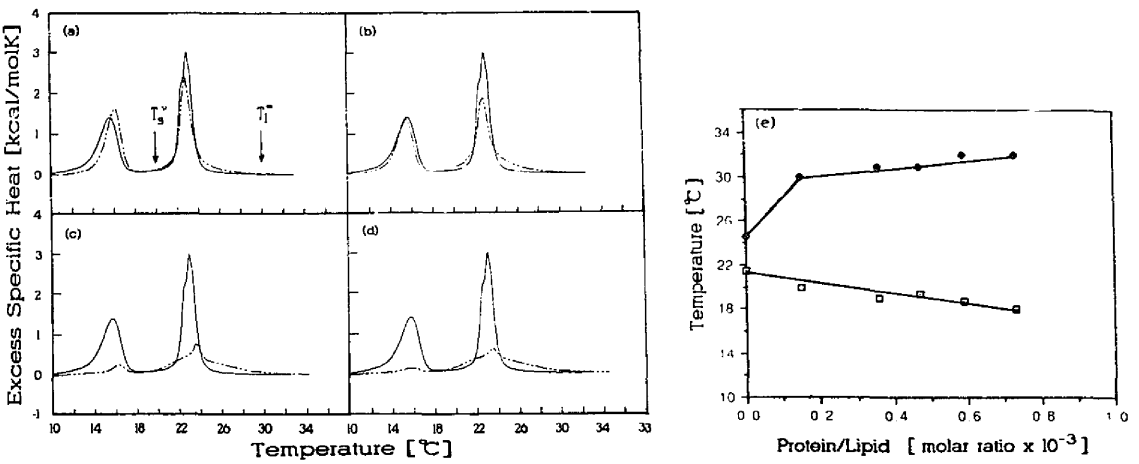

Fig. 5. DSC-measurements of pure and platelet talin-reconstituted mixed lipid vesicles. The left part of Fig. 5 shows the variation of pre- and main-phase transitions of mixed DMPC/DMPG vesicles in a ratio of 50:50 after incorporation of increasing concentrations of talin. The initial protein/lipid molar ratios added to the vesicle suspensions before freeze-thawing were: (a) $x^0_{p/l} = 1:6700$ $(1.5 \cdot 10^{-4})$; (b) $x^0_{p/l} = 1:2800$ $(3.6 \cdot 10^{-4})$; (c) $x^0_{p/l} = 1:1700$ $(5.8 \cdot 10^{-4})$ and (d) $x^0_{p/l} = 1:1400$ $(7.1 \cdot 10^{-4})$. (e) shows the shifts of the solidus lines ($\Delta T_s = T_s^* - T_s$), ⊡ and the liquidus line ($\Delta T_l = T_l^* - T_l$), ◆ as a function of the protein/lipid molar ratio.

hydrophobic interaction of platelet talin with DMPC vesicles, resulting in changes of heat and transition enthalpy simultaneously.

DMPC / DMPG (50 : 50) vesicles

Fig. 5 shows the results concerning the interaction of platelet talin with mixed vesicles of an equimolar mixture of DMPC and DMPG. Four specific heat vs. temperature plots for vesicles containing increasing amounts of platelet talin are presented. The initial protein/lipid molar ratios added to the vesicle suspensions prior to the freeze-thaw procedure were $x^0_{p/l} = 1:6700$ $(1.5 \cdot 10^{-4})$, $x^0_{p/l} = 1:2800$ $(3.6 \cdot 10^{-4})$, $x^0_{p/l} = 1:1700$ $(5.8 \cdot 10^{-4})$ and $x^0_{p/l} = 1:1400$ $(7.1 \cdot 10^{-4})$ for Figs. 5a, b, c and d, respectively. Density gradient centrifugation revealed only one sharp band corresponding to protein loaded vesicles (cf. also Fig. 3).

Measurements of the protein concentrations in the vesicle fraction and in the bottom fraction showed that all protein was incorporated into the vesicles and no unbound protein could be found in the bottom fraction (Table I).

The DSC plots (cf. Fig. 5) show the following essential features:

(1) The equimolar mixture of DMPC and DMPG exhibit remarkable sharp main- and pretransitions. This has to be explained in terms of complete miscibility of the two components in the L_α-, $P_{\beta'}$- and $L_{\beta'}$-phase of the mixtures. As a consequence the $P_{\beta'}$-phase exhibits a nearly defect-free ripple phase

TABLE I

Percentage of P-235 (platelet talin) reconstituted into vesicles with different lipid and protein compositions

Listed are: (1) the initial protein/lipid molar ratio; (2) the percentage of incorporated protein after five freeze-thaw cycles; (3) shift, ΔT_l^*, (liquidus line); (4) shift, ΔT_s^*, (solidus line).

	DMPC	DMPC/DMPG				DMPC/DMPS	
	100	70:30		50:50		70:25	
Initial protein/lipid molar ratio ($\times 10^{-4}$)	2.9	2.2	7.1	1.5	7.1	2.2	4.3
Percent of protein incorporated	37	72	77	100	100	69	70
$\Delta T_l = T_l^* - T_l$	≈ 0	5	5	5.5	7.5	6.5	8.5
$\Delta T_s = T_s^* - T_s$	≈ 0	1	2	1.5	3.5	–	–

with a well-defined wavelength as demonstrated by freeze-fracture electron microscopy (Fig. 2).

(2) Incorporation of platelet talin shifts the endpoint of chain melting, i.e., the liquidus line T_l of the lipid mixture to high temperatures (T_l^*) and the onset of chain melting T_s, i.e., the solidus line to lower temperatures (T_s^*).

(3) Interestingly, the high temperature shift of T_l^* exhibits saturation behaviour, i.e., the shift $\Delta T_l = T_l^* - T_l$ is only weakly dependent on the protein concentration. In contrast, the shift $\Delta T_s = T_s^* - T_s$ increases linearly with increasing protein concentrations (Fig. 5).

(4) The pronounced pretransition is only weakly shifted, but is gradually suppressed with increasing protein concentrations.

DMPC / DMPG (70 : 30) vesicles

In contrast to the 1 : 1 mixture of DMPC and DMPG but similar as with pure DMPC bilayers two populations of vesicles appear: a protein free and a protein loaded fraction. Analysis of small aliquots of the two vesicle bands (V_I and V_{II}) and of the bottom fraction of density gradients showed that the upper band (V_I) contained no protein, whereas a large portion of platelet talin was incorporated into the lower and heavier vesicle fraction V_{II} (see Fig. 3). A small amount of unbound platelet talin, however, was retained in the bottom fraction. Mixtures with an initial protein/lipid molar ratio of $x^0_{p/l} = 1:4500$ ($2.2 \cdot 10^{-4}$) and $x^0_{p/l} = 1:1400$ ($7.14 \cdot 10^{-4}$) were studied. At $x^0_{p/l} = 1:4500$, 72% of the protein appeared in vesicle fraction V_{II}. At $x^0_{p/l} = 1:1400$ only a slightly higher percentage (77%) was incorporated (Table I). This strongly suggests that the lipid/protein self-assembly exhibits saturation behaviour. As

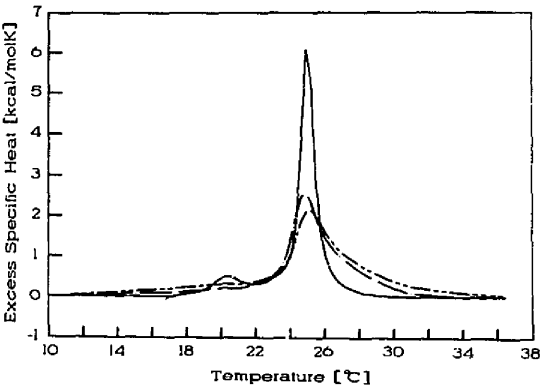

Fig. 6. Specific heat vs. temperature plots of mixed DMPC/DMPS (75 : 25) vesicles. Without protein (———) and with protein/lipid molar ratios of $x^0_{p/l} = 1:4500$ (·–·–·) and $x^0_{p/l} = 1:2300$ (– – – – –).

with a 1 : 1 mixture, the high-temperature shift of the liquidus line exhibits saturation behaviour and is $\Delta T_l = T_l^* - T_l \approx 6\,°C$, while the solidus line is continuously shifted to lower temperatures with increasing protein concentrations.

DMPC / DMPS (75 : 25) vesicles

Fig. 6 shows the modification of the phase change of mixed vesicles DMPC/DMPS for two different protein/lipid ratios: $x^0_{p/l} = 1:4500$ ($2.2 \cdot 10^{-4}$) and $x^0_{p/l} = 1:2300$ ($4.3 \cdot 10^{-4}$). Density gradient centrifugation resulted two sharp vesicle bands. The lighter, top one, was protein free. Phosphate determination and protein assays of vesicle band V_{II} and the bottom fraction showed that 69% of platelet talin was incorporated at $x^0_{p/l} =$

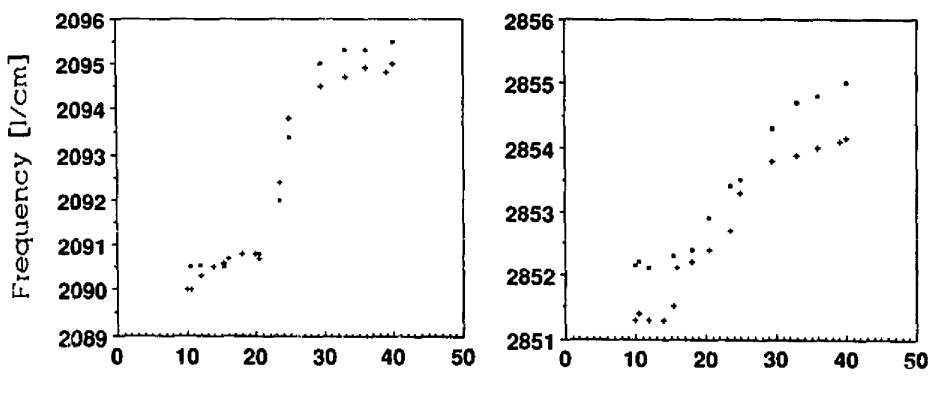

Fig. 7. FTIR study of the interaction of platelet talin with 1 : 1 mixed vesicles of chain-deuterated DMPC and DMPG. The initial protein/lipid molar ratio was $x^0_{p/l} = 1:2000$ ($5 \cdot 10^{-4}$). The temperature dependence of the symmetric C–D-stretching vibration of DMPC-d_{54} of pure lipid mixture (+) and of proteo-vesicle s(■) is shown on the left side. The temperature dependence of C–H-vibration of DMPG in the absence (+) and the presence (■) of platelet talin is shown on the right side.

1 : 4500 and 70% at $x^0_{P/l} = 1 : 2300$. This again shows that the lipid/protein self-assembly exhibits saturation behaviour. In analogy to DMPC/DMPG mixtures, both the main- and the pretransition are rather sharp showing that the charged and the zwitterionic lipids are completely miscible in the L_α-, $P_{\beta'}$- and $L_{\beta'}$-phase. Incorporation of platelet talin results in a large temperature shift of the liquidus line and a low temperature shift of the solidus line. However, in this case the shift of the liquidus temperature depends remarkably on the protein concentration. The low-temperature shift of T_s^* cannot be determined exactly due to the overlaps of the $L_{\beta'}$–$P_{\beta'}$-transition.

FTIR-spectroscopy

To gain further insight into the selective lipid-protein interaction, FTIR spectra with one of the lipid components deuterated were taken in the absence and the presence of platelet talin. Spectra are plotted in Fig. 7 for a 1 : 1 mixture of chain-deuterated DMPC-d_{54} and non deuterated DMPG showing the symmetric C–D-stretching vibrational band of DMPC-d_{54} in the 2090 cm^{-1} region and the C–H-stretching band of DMPG. For the pure lipid mixture, the phase changes in the regions of the main- and the pretransitions are well indicated by abrupt changes in the position of the band. Interestingly, the pretransition is much more pronounced for DMPG than for DMPC. The two phase transitions are rather sharp, which again shows that the mixture behaves nearly ideally.

Incorporation of platelet talin affects the spectrum for DMPG much stronger than that for DMPC. First, in the fluid state the bands of both components are shifted to shorter wavelengths, but the relative shift is considerably larger for DMPG than for DMPC. Second, the position and width of the main transition of the non-charged component are only slightly changed by the protein, while for the charged component, T_l is shifted to $T_l^* = 33°C$ and the solidus temperature T_s^* is shifted to about 18 °C. Both shifts are in reasonable agreement with the calorimetric data. In summary, the FTIR data suggest a prominent selectivity for a platelet talin-DMPG interaction. In the limit of infinite dilution the distribution ratio should not depend on the initial concentrations of the constituents.

Membrane bound and cytoplasmic platelet talin interact differently with DMPC/DMPG bilayers

Fig. 8 presents specific heat vs. temperature plots of a DMPC/DMPG (70 : 30) mixture and the modifications of phase changes ($P_{\beta'}$–L_α and $L_{\beta'}$–$P_{\beta'}$-transition) induced by platelet talin purified (i) from a Triton-lysed, membrane-containing fraction and (ii) from a cytoplasmic pool. For both fractions the high-temperature shift, $\Delta T_l = T_l^* - T_l$, is about equal and the pretransition is clearly suppressed. The cytoplasmic fraction of platelet talin, however, does not cause a remarkable shift of the solidus line! This difference suggests that the cytoplasmic fraction of platelet talin cannot penetrate into the hydrophobic region of the membrane, but interacts only electrostatically with the bilayer surface.

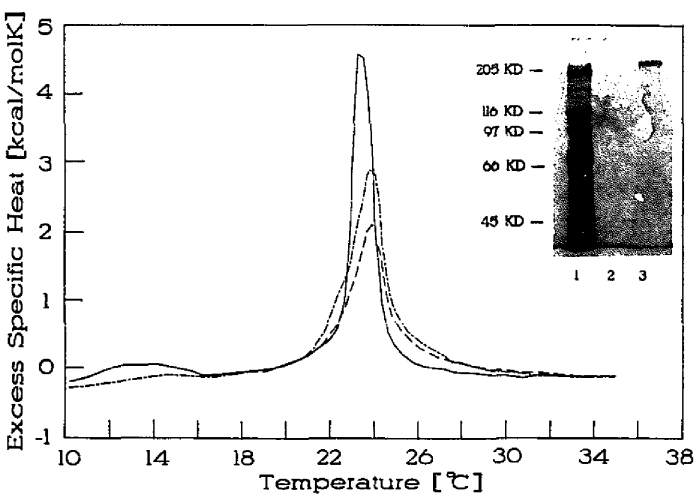

Fig. 8. Reconstitution of membrane bound and cytoplasmic platelet talin with lipid bilayers. DSC-analysis of reconstituted platelet talin purified from the cytoplasmic pool (- - - - - -) and from a Triton-lysed membrane containing fraction (- - - -) in comparison with controls (———). Mixed DMPC/DMPG (70 : 30) vesicles with protein/lipid molar ratios of $x^0_{P/l} = 1 : 2800$ ($3.6 \cdot 10^{-4}$) were used. The inset shows silver-stained SDS-gels (7.5%) of density gradient fractions of a vesicle/protein mixture treated with 250 mM NaCl for 1 h at 30 °C. Under these conditions cytoplasmic platelet talin does not incorporate into the vesicle fraction (lane 2), but is salted out and retained in the bottom fraction, lane 3. Protein standards, lane 1.

Evidence for this interpretation was provided by the finding, that purified cytoplasmic platelet talin when added to mixed DMPC/DMPG (70:30) vesicles in the presence of 250 mM NaCl was salted out and did not reconstitute into the vesicle fraction (Fig. 8, inset). In contrast, incubation of proteo-vesicles containing platelet talin from the Triton-lysed membrane fraction with up to 500 mM NaCl did not displace the protein from vesicles at all, showing that this protein form is stably anchored in the bilayer.

Decoupling of the electrostatic binding domain of platelet talin by NaCl

Fig. 9 shows the effect of high salt concentrations (500 mM NaCl) on the interaction of the amphiphilic (Triton-isolated) fraction of platelet talin with vesicles containing charged lipids. After NaCl treatment, the main transition of proteo-vesicles no longer exhibits a high-temperature shift of the liquidus line ($T_l^* = T_l$), whereas the solidus line is still shifted to lower temperatures. As in untreated samples, the heat of the pretransition is slightly reduced again. Both phenomena indicate that the electrostatic coupling is shielded by the salt treatment, whereas the hydrophobic interaction still exists. As a consequence, platelet talin under these conditions is still anchored to the membrane, as can be demonstrated by SDS-gels of the bottom fraction and the vesicle band (V_{II}) obtained after density gradient centrifugation (data not shown). Hence, after high salt incubation and electrostatic decoupling platelet talin purified from a Triton-lysed membrane fraction is able to bind to the lipid bilayer, probably by its hydrophobic domain.

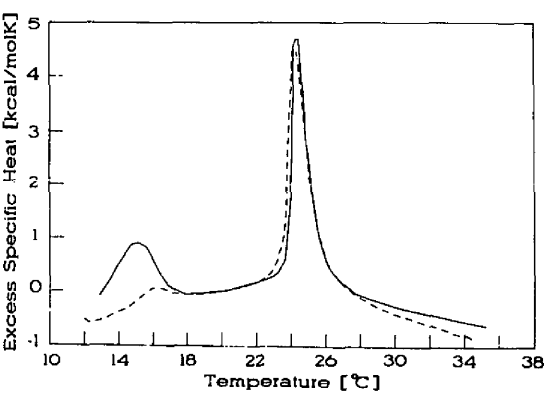

Fig. 9. Effect of NaCl on the interaction of platelet talin with DMPC/DMPG (1:1) mixed vesicles. DSC, profile of the pure lipid mixture after incubation with 500 mM NaCl for 1 h at 30°C (------). DSC profile of proteo-vesicles with a protein/lipid molar ratio of 1:2800 ($3.6 \cdot 10^{-4}$) after identical salt treatment (———).

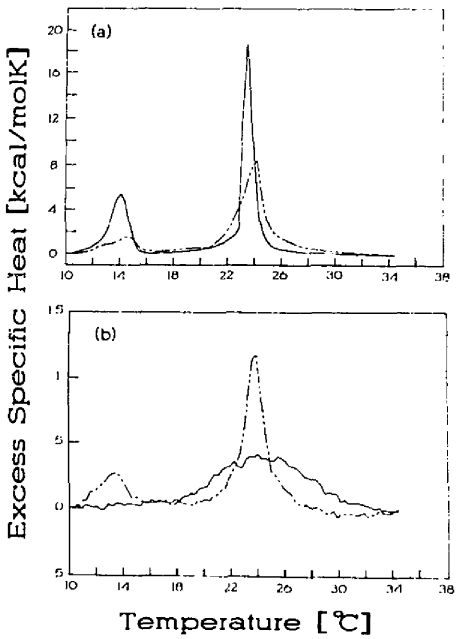

Fig. 10. Comparison of specific heat vs. temperature plots of reconstituted platelet talin in DMPC/DMPG (70:30) vesicles prior to and after density gradient centrifugation. (a) DSC plots of DMPC/DMPG vesicles without protein (———) and with reconstituted platelet talin in a molar ratio $x^0_{p/L} = 1:2800$ ($3.6 \cdot 10^{-4}$) prior to gradient centrifugation (--------). (b) Thermogram of proteo-vesicles of fraction V_{II} prepared by density gradient centrifugation (———). The same fraction after pronase treatment (--------).

Treatment of proteo-vesicles with pronase and thrombin

To ascertain that the shift of DSC-profiles results from the incorporation of right-side-out oriented platelet talin in the vesicle membrane, we have treated reconstituted vesicles with pronase. The two vesicle fractions V_I and V_{II} were first separated by density gradient centrifugation, dialysed over night to remove the metrizamide and incubated with pronase for 2 h at a pronase to protein ratio of 1:10. Fig. 10 shows DSC plots of vesicles before and after density gradient centrifugation, as well as the influence of pronase on the transition behaviour of the reconstituted vesicle fraction. The main- and pretransition, which appear largely reduced in a mixed fraction of reconstituted and protein-free vesicles (Fig. 10a) and have almost vanished in the proteo-vesicle fraction obtained after density gradient centrifugation, become visible again as two distinct peaks (Fig. 10b) after pronase treatment. This clearly demonstrates that the lipid–protein interactions as monitored by the transition behaviour change quite dramatically when pronase is applied exogenously. Since the treatment with pronase leads to a complete protein degradation (gel data not shown), platelet talin is not

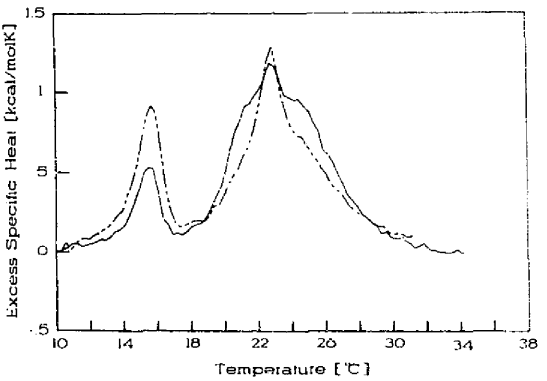

Fig. 11. Thrombin induced cleavage of platelet talin reconstituted with a protein/lipid molar ratio of $x^0_{p/L} = 1:1400$ ($7 \cdot 10^{-4}$) into 1:1 DMPC/DMPG vesicles. Specific heat vs. temperature plot obtained before (———) and after (- - - - - - - -) a 12 h incubation with 0.1 units of thrombin.

trapped inside the vesicles, but reconstitutes into the outer monolayer with an outside orientation.

In line with these results and like those in vivo, reconstituted platelet talin can still be cleaved by thrombin. Fig. 11 shows thermograms obtained for reconstituted vesicles before and after thrombin incubation. After thrombin treatment the two pre- and main transition peaks can clearly be recovered again, which indicates that most of the protein has been decoupled from the membrane after cleavage into its specific subunits.

Distribution of platelet talin between the aqueous and the membrane phase

Phosphate analysis and the determination of unbound and reconstituted protein concentrations yields a distribution ratio of the protein between the aqueous phase and the membrane. This ratio (x_w/x_m) is given in Table II for various lipid mixtures, where x_m and x_w represent the protein molar fractions in the proteovesicles (V_{II}) and water. Provided that the redistribution of platelet talin between these two phases during freeze-thawing is an equilibrium process, the Nernst distribution law

$$x_w/x_m = \exp\{-(\mu_w - \mu_m)/kT\}$$

may be applied, where μ_w and μ_m are the chemical potentials of the protein in the aqueous phase and the membrane. The law predicts that the ratio does not depend on the initial protein/lipid molar ratio $x^0_{p/l}$. The data in Table II indeed provide evidence that the Nernst distribution is fulfilled.

Discussion

By choosing appropriate lipids, proteins can be reconstituted into vesicles by cyclic freezing and re-thawing of suspensions of (sonicated) vesicles and solubilised protein. Remarkably, the freeze-thaw technique yields a uniform orientation of platelet talin with its large head group pointing to the exterior of the vesicles (right-side-out orientation). Similarly oriented, the platelet fibrinogen receptor was shown to reconstitute into mixed PC-PS vesicles by dialysis [17]. The freeze-thaw procedure used for our experiments, however, has the advantage that a contamination with a tenside, which in most cases is necessary for the solubilisation of protein and lipid, can be neglected.

In Table I, we have summarised the results concerning the fractions of protein incorporated into the vesicles and the shifts of the liquidus lines ($\Delta T_l = T_l^* - T_l$) and the solidus lines ($\Delta T_s = T_s^* - T_s$). In the following we wish to discuss some consequences from these data.

(1) Platelet talin (P-235) can be reconstituted into vesicles composed of zwitterionic lipids alone; however, the addition of charged lipids increases the solubility of

TABLE II

Distribution of P-235 (platelet talin) between the aqueous phase and the membrane given in terms of the ratio of protein molar fraction in the aqueous phase (note: 1 ml of water = 5.6·10^{-2} mol) and the membrane fraction V_{II} (proteo-vesicles)

$x^0_{p/l}$ represents the initial protein/lipid molar ratio in suspension prior to freeze-thawing. x_w/x_m represents the ratio of protein molar fractions in the aqueous (x_w) and the membrane (x_m). Differences of chemical potentials in the aqueous phase and the membrane are obtained by the Nernst distribution law.

Lipid mixture	$x^0_{p/l}$ = ($\times 10^{-4}$)	Protein in membrane (nM)	Protein in water (nM)	total lipid (nM)	x_w/x_m ($\times 10^{-6}$)	$\mu_w - \mu_m$ (units of k_BT)
DMPC	2.9	0.081	0.14	826	25.60	−10.57
DMPC/DMPG	2.2	0.183	0.0272	715	5.02	−12.20
(70:30)	7.1	0.700	0.21	732	3.92	−12.45
DMPC/DMPG	1.5	0.155	0	540	–	–
(50:50)	7.1	0.370	0	550	–	–
DMPC/DMPS	2.2	0.310	0.14	750	6.05	−12.02
(70:30)	4.3	0.200	0.09	600	4.81	−12.24

the protein in membranes drastically. Thus, at 50% of DMPG the protein is completely incorporated into vesicles.

(2) The persistence of main- and pretransitions, which are masked after protein incorporation but become clearly evident again after proteolytic digests, shows that the protein, when moving into the lipid bilayer does not change the lipid composition of the vesicle. This is an interesting finding, since it rules out a segregation of lipid components as a consequence of protein reconstitution and hence is essential for the preparation of proteo-vesicles with a controlled lipid composition. Furthermore, it provides evidence that the assembly of proteo-vesicles by freeze-thawing indeed is an equilibrium process.

(3) With pure DMPC vesicles we did not find a remarkable shift of the phase transition, although the density centrifugation and protein assays clearly showed that protein-loaded vesicles were formed. An interaction of platelet talin with DMPC, however, was revealed by the linear decrease of the heat of transition, ΔH, with increasing protein concentration. At low protein concentrations ($x_p \approx 10^{-3}$) the variation of the heat of transition, $\Delta H_{m,L}$ of the lipid is given by

$$\Delta H_{m,L} = \Delta H^0_{m,L} - (h^{ex}_{\alpha} - h^{ex}_{\beta}) \cdot x_p \qquad (1)$$

where $\Delta H^0_{m,L}$ is the heat of transition of the pure lipid, while h^{ex}_{α} and h^{ex}_{β} represent the partial excess heats of the mixture in the L_{α} and $P_{\beta'}$-phases, respectively. On the other side, the shift of the chain-melting temperature is (in the limit of infinite dilution)

$$\Delta T = T_m - T^0_m = (RT_m/\Delta H^0_m) \cdot \ln(x^{\alpha}_L/x^{\beta}_L) \qquad (2)$$

The absence of any shift ΔT for DMPC shows that the solubility of the protein in the crystalline phase is high and that DMPC-talin forms an ideal solid solution.

(4) The preferred interaction of platelet talin with negatively charged lipids is impressively demonstrated by the FTIR spectra. The position of the C–H-stretching vibrational band ν_0 is a rough measure for the relative number of C-atoms in a *gauche* conformation. The main- and the pretransition of the pure lipid mixture are clearly exhibited by the spectra of both lipids. The sharpness of the transition shows again that the DMPC-DMPG mixture behaves nearly ideal.

In the fluid state, platelet talin causes a high-frequency shift, $\Delta\nu$, of both lipids. The relative displacement $\Delta\nu/\nu$ is, however, considerably larger for DMPG, which is for the first time an indication that platelet talin interacts more strongly with this lipid than with DMPC. Further evidence for such a selective interaction is provided by the finding that the two lipids behave remarkably differently in the main phase transition region: the band shift of the zwitterionic lipid with temperature is hardly changed by the protein. In contrast, the transition of DMPG is strongly broadened and the position of the phase boundaries T_s^* and T_l^* agree well with those shown by the DSC profiles. This shows that the high temperature shift of the liquidus line is determined by DMPG, which leads to the conclusion that the temperature shift is caused by an electrostatic interaction of platelet talin specifically with this lipid. Since the shift ΔT_l^* is essentially determined by the logarithm of the ratio of the protein concentrations in the fluid and solid lipid phases (cf. Eqn. 2 and note that the protein concentration is very low) the large value of ΔT_l^* suggests that upon cooling below T_l^* phase separation into a protein-free and DMPG-rich solid phase and a fluid DMPC and protein-rich phase occurs. Thus, in this temperature range the three component system (lipid-lipid-protein) exhibits a pronounced non-ideality.

(5) Evidence for a talin-DMPG-complex formation or protein induced phase separation in the fluid (L_{α}) phase is obtained by the remarkable finding that the high temperature shift of the liquidus line, ΔT_l, increases sharply with increasing protein concentration and is saturated at about $x_p \leqslant 2 \cdot 10^{-4}$ in the case of a 1:1 DMPC/DMPG mixture (cf. Fig. 5d) and at even lower protein concentrations in the case of the 70:30 mixture (cf. Table I). Moreover, the shift is nearly the same for both mixtures. This provides evidence that a defined number of DMPG molecules interact with the protein which could be interpreted as PG-talin complexes, or in the terminology of colloid chemistry as phase separation in the fluid state. The saturation concentration would then correspond to the phase boundary of the miscibility gap of the fluid lipid/protein mixture.

(6) As we have found, platelet talin seems to exist in two modifications. One form interacts electrostatically and hydrophobically with charged bilayers and obviously exists in a membrane bound fraction already in vivo. The other form of platelet-talin seems to interact only electrostatically with charged lipids and is found in the cytoplasm. Since platelet talin after activation of platelets moves from the cytoplasm towards the plasma membrane [11], it is reasonable to speculate that cytoplasmic talin is converted to a form which is able to interact with membrane lipids, possibly by a post-translational modification such as acylation or myristilation.

(7) Concerning the work of transfer of protein from the aqueous phase to the membrane the data from Table II can be used, where we have determined the ratio x_w/x_m, i.e., the molar fraction of platelet talin in the aqueous phase and in the membrane. We also noted that provided the lipid-protein assembly by freeze-thawing is an equilibrium process it is possible to determine the chemical potential difference

$$\Delta\mu = \mu_w - \mu_m$$

which is a measure for the work per protein molecule associated with the transfer of one protein molecule from the aqueous phase into the membrane. It was also noted above that evidence for the assembly as an equilibrium process derives from the finding that x_w/x_m does not depend on the initial lipid and protein concentrations.

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