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Lipid-Membrane Affinity of Chimeric Metal-binding Green Fluorescent Protein

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Abstract. The Green Fluorescent Protein (GFP) is a useful marker to trace the expression of cellular proteins. However, little is known about changes in protein interaction properties after fusion to GFP. In this study, we present evidence for a binding affinity of chimeric cadmium-binding green fluorescent proteins to lipid membrane. This affinity has been observed in both cellular membranes and artificial lipid monolayers and bilayers. At the cellular level, the presence of Cd-binding peptide promoted the association of the chimeric GFP onto the lipid membrane, which declined the fluorescence emission of the engineered cells. Binding affinity to lipid membranes was further investigated using artificial lipid bilayers and monolayers. Small amounts of the chimeric GFP were found to incorporate into the lipid vesicles due to the high surface pressure of bilayer lipids. At low interfacial pressure of the lipid monolayer, incorporation of the chimeric Cd-binding GFP onto the lipid monolayer was revealed. From the measured lipid isotherms, we conclude that Cd-binding GFP mediates an increase in membrane fluidity and an expansion of the surface area of the lipid film. This evidence was strongly supported by epifluorescence microscopy, showing that the chimeric Cd-binding GFP preferentially binds to fluid-phase areas and defect parts of the lipid monolayer. All these findings demonstrate the hydrophobicity of the GFP constructs is mainly influenced by the fusion partner. Thus, the example of a metal-binding unit used here shines new light on the biophysical properties of GFP constructs.

Key words: Green fluorescent protein — Lipid-binding — Cd-binding peptide — Lipid bilayers — Lipid monolayers

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Introduction

Green fluorescent protein (GFP) is an autoilluminating protein isolated from the jellyfish, *Aequorea victoria*. It is a relatively small monomeric protein composed of 238 amino acids with molecular mass of 29 KDa (Shimomura, Johnson & Saiga, 1962; Morise et al., 1974; Shimomura, 1979). It has a major excitation peak at 395 nm and a minor peak at 470 nm with a single emission peak at 509 nm (Chalfie et al., 1994). The GFP expression is species-independent and requires no substrates or cofactors for the fluorescence formation. The fluorescence is generated by an internal chromophore via spontaneous posttranslational oxidation of residues Ser^{65}, Tyr^{66}, and Gly^{67} within a hexapeptide at position 64–69 (Cody et al., 1993). The chromophore is amazingly resistant to a wide variety of hazardous conditions including high temperature, extreme pH, and proteases (Bokman & Ward, 1981). Even under drastic acidic/basic conditions or highly potent denaturants, e.g. 6 M guanidine hydrochloride or 8 M urea, GFP regains its natural fluorescence after removal of the drastic condition (Bokman & Ward, 1981; Ward & Bokman, 1982). In the present study, we used a GFP variant (GFPuv), which was optimized for UV excitation and emitting 18 times more fluorescence intensity than wild-type GFP. This variant GFP can also easily be detected by irradiation with standard long-wave UV or blue light (Crameri et al., 1996).

Due to the autofluorescent property of GFP, much attention has been focused on applying the GFP as a fusion partner to monitor gene expression and protein localization in both prokaryotic and eukaryotic cells (Chalfie et al., 1994; Cormack, 1998; Inouye & Tsuji, 1994a; Kain et al., 1995; Hampton et al., 1996; Welsh & Kay, 1997; Cha et al., 1999a). In addition, GFP has also been used for investigation of protein-protein interactions (Garamszegi et al., 1997). Because of the variety of applications of GFP,

the fluorescence properties of this molecule have been improved and the physical and chemical effects on fluorescence emission have been extensively explored (Bokman & Ward, 1981; Inouye & Tsuji, 1994b; Yang, Cheng & Kain, 1996; Kimata et al., 1997; Kojima et al., 1997; Yang et al., 1998). However, more knowledge at the molecular level, particularly on binding to other biomolecules, needs to be discovered to allow the design of molecular fluorescent proteins for a variety of applications.

We have constructed a series of chimeric genes encoding chimeric GFP carrying a variety of metal-binding peptides including the chimeric GFPs having hexapolyhistidine or Cd-binding peptide. Such engineered chimeric GFPs have been applied as a potential tool for metal determination both at the purified protein and at the cellular level (Isarankura Na Ayudhya, 2000; Prachayasittikul et al., 2000; Prachayasittikul, Isarankura Na Ayudhya & Bulow, 2001). The Cd-binding peptide (His-Ser-Gln-Lys-Val-Phe) is composed of hydrophobic amino acids flanked by basic amino acids in tandem repetition (Mejare, Ljung & Bulow, 1998). In this study we used these Cd-binding peptides as tools to study the association of chimeric green fluorescent proteins to lipid membranes. Evidences of chimeric proteins binding to cellular as well as artificial lipid membrane are presented. Our present findings, using one class of fusion peptides presently under consideration for metal sensing, may also apply to other chimeric GFP-proteins.

Materials and Methods

Bacterial Strain and Plasmids

Escherichia coli (*E. coli*) strain TG1 (lac-pro), Sup E, thil, hsd D5/F' tra D36, pro A^+ B^+, lacI, lacZ, M15; (ung^+, dut^+) was used as host. Plasmids pHis6GFPuv (Prachayasittikul et al., 2000) and pCdBP_4GFPuv (Prachayasittikul et al., 2001) were used for construction.

Lipids, Chemicals and Biological Reagents

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoserine (DPPS), l,2-dipalmitoyl-sn-glycero-3-(phospho-rac-(1-glycerol)) (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS) were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Solvents were high performance liquid chromatography grade and purchased from Merck (Darmstadt, Germany). Water was first purified through a millipore water purification system Milli-Q RO 10 Plus (Millipore GmbH, Eschborn, Germany) and then finally with the millipore ultrapure water system Milli-Q Plus 185 (18.2 MΩ cm^{-1}). For all experiments, a PBS (50 mM Na_2HPO_4, 0.3 M NaCl, pH 7.4) was used, if not stated otherwise. Lipid stock solutions were made by dissolving powdered lipid in chloroform or chloroform/methanol at appropriate molar ratio (1:1 or 1:3).

Restriction endonucleases, T4 DNA ligase and molecular weight marker (λ/*Hind*III) were obtained from New England Biolabs. Chelating Sepharose Fast Flow gel was purchased from Pharmacia Biotech, Sweden.

Chimeric Gene Construction

Cloning procedures were performed as described by Maniatis et al. (Maniatis, Fritsch & Sambrook, 1989). To construct a chimeric green fluorescent protein having a combination of hexahistidine and four Cd-binding regions as the metal-binding site, the gene encoding Cd-binding regions fusing to GFP was cleaved out from the pCdBP4GFPuv, then ligated into the *Sac*I site of the pHis6GFPuv. The ligation product was subsequently transformed into *E. coli*. Transformants were selected and the inframe-fusing of chimeric gene was checked via restriction endonuclease analysis. The chimeric gene was subsequently expressed in *E. coli* strain TG1. Gene expression was readily monitored by following the cell fluorescence.

Protein Preparation and Purification

Both native and chimeric green fluorescent proteins were harvested from cultures of *E. coli* TG1 carrying constructed plasmids. Briefly, the culture was spun at 10,000 × *g* for 5 min and the cell pellet was resuspended in 50 mM sodium phosphate buffer, pH 7.4, containing 0.3 M NaCl. Cells were disrupted by sonic disintegration (sonicator ultrasonic processor model XL, Heat System Incorporation, USA) at output 6 for 20 s (six times) with resting of 40 s in between and debris was removed by centrifugation (10,000 × *g*, 5 min). The supernatant was attained as crude constructed protein preparation.

In the cases of CdBP_4GFP and His6CdBP4GFP chimeric proteins, the majority of the constructed protein was associated with the debris fraction. Therefore, the pellet of cell debris of the centrifugation was collected. The chimeric proteins were released by resuspending in phosphate buffer containing 6 M guanidine hydrochloride. The sample was clarified by spinning at 10,000 × *g* for 5 min at 4°C prior to further purification by the IMAC-Zn affinity chromatography as previously described (Prachayasittikul et al., 2000).

Fluorescence Measurements

Fluorescence was assayed by irradiation of either the purified GFPs or the engineered cells at 395 nm and subsequent emission of photons at 509 nm was recorded via fluorescence multi-well plate readers (BIOTEK, USA and BMG Labtechnologies, FRG).

Determination of Binding Capacity of Chimeric Metal-binding GFPs to Multilamella Vesicles (MLVs)

Binding capacity of chimeric His6CdBP4GFP to liposomes was determined as compared to the chimeric His6GFP. Briefly, multilamella vesicles (MLVs) of pure DPPC/DOPC or lipid mixtures (DPPC:DPPS/DPPG/DPPA; 4:1) were prepared. Aliquots of lipids in a small glass tube were evaporated to dryness under a stream of nitrogen and then under high vacuum. PBS was added and the lipid was dispersed above the phase-transition temperature of each lipid by vortexing for 30 s (3–4 times). The MLVs (100 μg) were incubated with the chimeric GFPs (10 μg) at room temperature for an hour followed by centrifugation at 10,000 × *g* at 4°C for 15 min. The pellets were then washed with the buffer to remove unbound protein. The supernatants were collected and the remaining pro-

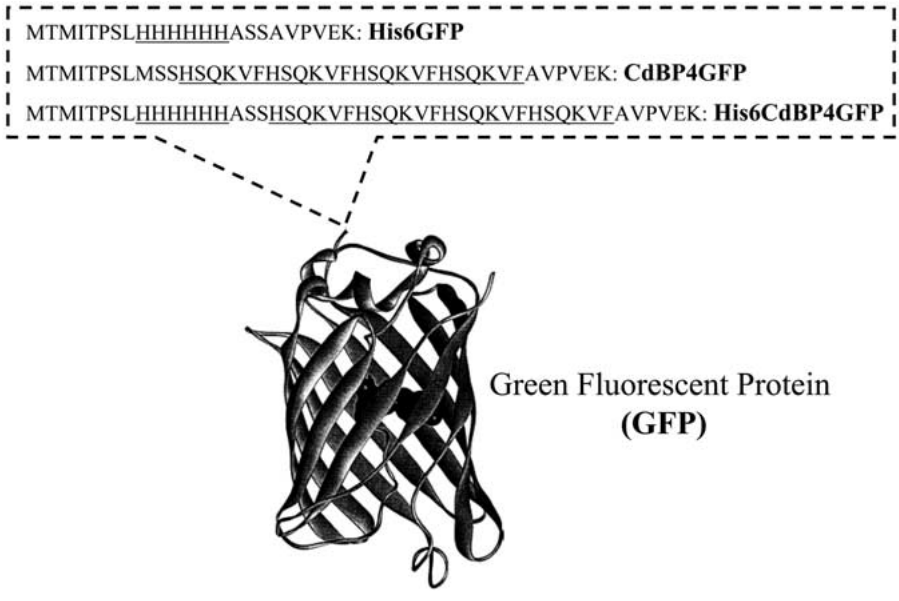

Fig. 1. Schematic representation of chimeric green fluorescent protein carrying hexahistidine (*His6GFP*), peptide with four Cd-binding regions (*CdBP4GFP*) or peptide with hexahistidine-four-Cd binding regions (*His6CdBP4GFP*).

teins were precipitated using 20% trichloroacetic acid (TCA). Finally, both fractions were analyzed on SDS-PAGE.

Film-Balance Measurements

Measurements were performed on a Wilhelmy film balance (Riegler and Kirstein, Mainz, Germany) with an operation area of 40 cm^2 and a bulk volume of 24 ml PBS at a temperature of 20°C. The position and scanning speed of the film-balance barrier, as well as the recording of area–pressure isotherms, were computer controlled. Monolayers were composed of either DPPC or DOPC. Prior to each experiment, the trough and barrier were cleaned with mucasol™ and dichloromethane followed by rinsing with deionized water. Phospholipid films were spread from a chloroform solution with a microsyringe at the air/liquid interface. After an equilibration time of 10 min, the film was compressed with a constant compression rate (5.81 cm^2/min) until the final surface pressure reached 10 mN/m. The interface was allowed to equilibrate for a minimum of 30 min to maintain constant pressure. The subphase was gently and continuously stirred by a magnetic bar. The chimeric GFP dissolved in PBS was then injected into the subphase underneath the monolayer via an inlet port in the trough. Changes of the lateral pressure after injection were measured at constant surface area and recorded for a minimum of 60 min. In addition, the isotherms before and after protein injection were determined.

Epifluorescence Measurements

Fluorescence of the lipid monolayer (DPPC) doped with either His6CdBP4GFP or His6GFP was excited and visualized via an epifluorescence microscope (Olympus STM5-MJS, Hamburg, Germany). The Langmuir trough equipped with a computer-controlled movable barrier and a Wilhelmy system for measurement of the surface tension was placed on a specially designed stage (Riegler and Kirstein, Mainz, Germany) for the microscope. With the help of the remote-controlled stage, the trough could be moved independently in the three directions of the axes (x, y, z) of a Cartesian coordinate system where the x and y axes were oriented perpendicular to the optical axis of the objective lens. For excitation, a high-pressure mercury lamp with a power of 50 Watt was used. Discrimination of excitation light and emitted light of the green fluorescent protein was achieved by cut-off filters. To perform the experiment, drops of the lipid stock solution were formed on the end of a Hamilton syringe and carefully spread to the air-liquid interface. The solvent was allowed to evaporate for at least 10 min. After evaporation, the interface was compressed until the surface pressure reached 5 mN/m. Then, the chimeric GFP was injected into the subphase without disturbance of the lipid monolayer and the interface was further compressed to 10 and 35 mN/m, respectively. In parallel, the fluorescence at each pressure was detected using a SIT-camera (Hamamatsu, Hamamatsu, Japan).

Results

Construction and Expression of Chimeric Genes Encoding Chimeric Metal-binding Green Fluorescent Proteins

A series of chimeric genes encoding chimeric metal-binding green fluorescent proteins (chimeric GFPs) have successfully been constructed. These included chimeric genes of His6GFP encoding a hexapolyhistidine and green fluorescent protein (Prachayasittikul et al., 2000); CdBP4GFP encoding a peptide with four cadmium-binding regions and the green fluorescent protein (Prachayasittikul et al., 2001) and His6CdBP4GFP encoding a hexapolyhistidine tail, a peptide with four cadmium-binding regions and the green fluorescent protein (Fig. 1). Those proteins were primarily constructed to build up biosensor devices. Herein, however, they are used as tools to investigate the membrane-binding properties, which are important to know not only for this but also for other GFP-constructs used in biotechnology.

Engineered cells (*E. coli*) expressing all chimeric GFPs possessed fluorescence activity (Fig. 2*A*). However, fluorescence intensity at the cellular level varied and this was determined by spectrofluorome-

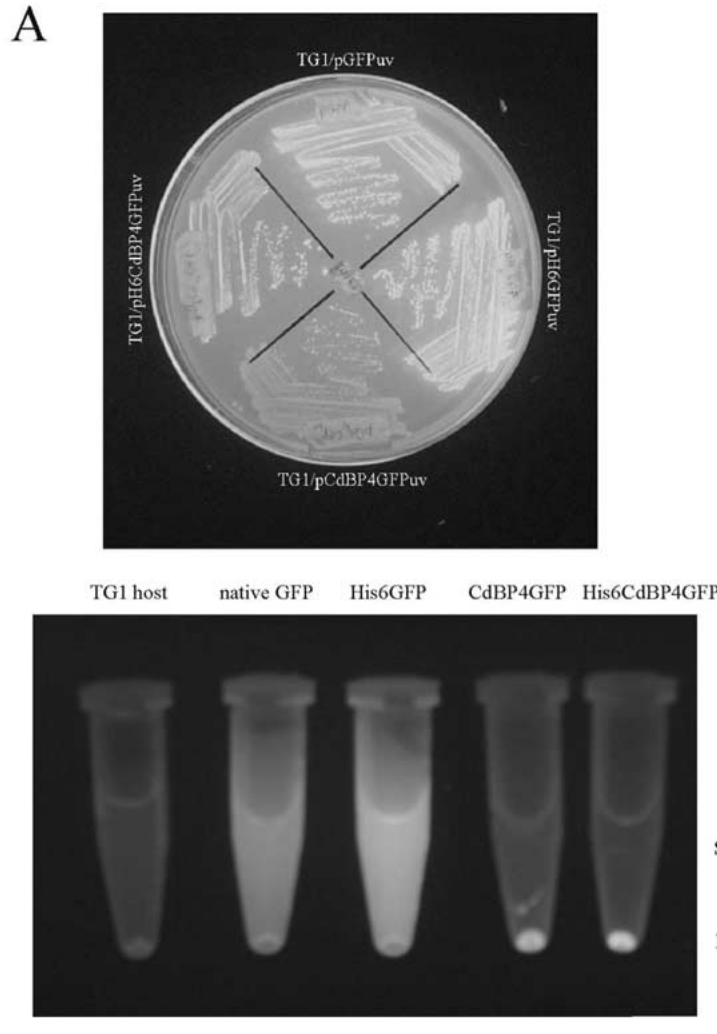

Fig. 2. (*A*) Fluorescent emission of engineered cell expressing native GFP, His6GFP, CdBP4GFP and His6CdBP4GFP. (*B*) Localization of chimeric proteins in various compartments of sonicated cells expressing native GFP, His6GFP, CdBP4GFP and His6CdBP4GFP.

try. The cells expressing chimeric His6GFP were approximately 3-fold higher in fluorescence activity (8,239 FU/10^7 cells) compared to cells expressing native GFP (2,454 FU/10^7 cells). The fluorescence of cells expressing CdBP4GFP was 1.6-fold (1,530 FU/10^7 cells) lower than those of cells with the native green fluorescent protein. The cells expressing His6CdBP4GFP provided the same fluorescence intensity level as CdBP4GFP (1,589 FU/10^7 cells).

Engineered cells expressing chimeric genes were fractionated. As shown in Fig. 2*B*, the native GFP was expressed and found in the cytosol, while the chimeric CdBP4GFP was found to be almost all associated with membranes, thus remaining in the debris fraction after centrifugation. The presence at the membranes and not in inclusion bodies has been proven by fluorescence microscopy. The chimeric His6GFP remained exclusively in the cytosol. Engineering of a hexahistidine into the four cadmium-binding sequences of the chimeric protein did not affect the hydrophobic association of CdBP4GFP to the membrane. Therefore, almost all of the His6CdBP4GFP was found in the cell debris after disruption of the cells by sonic disintegration and centrifugation (Fig. 2*B*).

Association of chimeric GFP to the cell compartment might subsequently affect the fluorescence at the cellular level. Therefore, fluorescence intensity of each purified chimeric GFP was further determined. The chimeric CdBP4GFP and the His6CdBP4GFP were extracted using guanidine hydrochloride. The chimeric proteins possessing dual characteristics of both metal binding and fluorescence emission were purified to homogeneity via immobilized metal affinity chromatography (IMAC) loaded with zinc ions. The integrity of recombinant protein was analyzed on SDS-PAGE. The increase in molecular weight of chimeric His6CdBP4GFP as compared to the original chimeric proteins (His6GFP and $CdBP_4GFP$) is obvious (Fig. 3*A*). The fluorescence intensity (FU/μg) decreased in the order of His6GFP (3,210 FU/μg) > CdBP4GFP (2,583 FU/μg) >

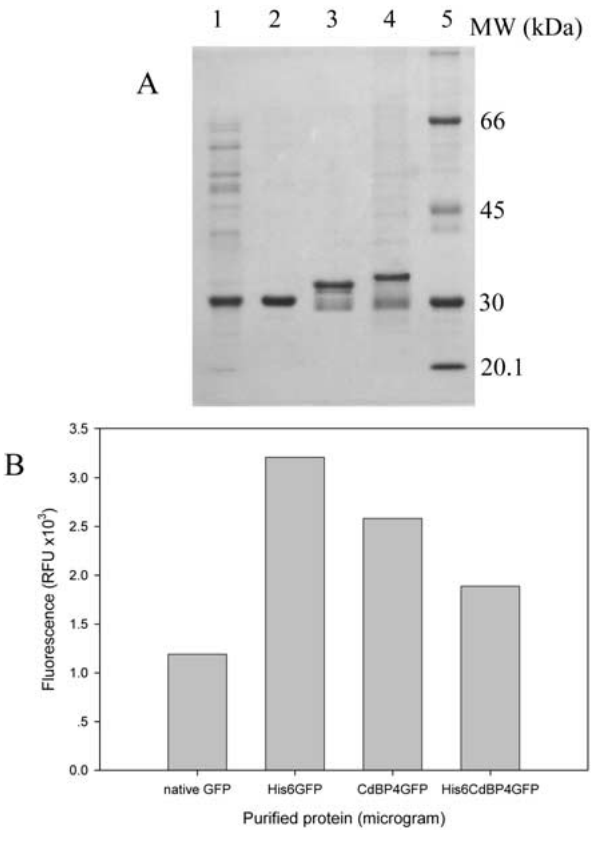

Fig. 3. (*A*) SDS-PAGE of chimeric metal-binding green fluorescent proteins. Lane *1*, native GFP; lane *2*, His6GFP; lane *3*, $CdBP_4GFP$; lane *4*, $His6CdBP_4GFP$; and lane 5, standard protein markers. (*B*) Specific fluorescence activity (RFU/μg) of purified chimeric GFPs.

His6CdBP4GFP (1,889 FU/μg) > native GFP (1,191 FU/μg), as represented in Fig. 3*B*.

Interaction of Chimeric Green Fluorescent Proteins with Artificial Membrane

Binding of Chimeric GFPs to Multilamellar Vesicles

To test whether the chimeric GFP consisting of the Cd-binding regions possessed an affinity for lipid membranes, the chimeric His6CdBP4GFP was incubated with multilamellar vesicles of either saturated or nonsaturated phospholipids with different head groups (e.g., DPPC, DPPS, DPPG, DPPA, DOPC or DOPS). Lipid-bound chimeric protein was then precipitated and subsequently analyzed on SDS-PAGE. This lipid-protein complex yields two bands in the SDS-gel with the molecular masses corresponding to the lipid and the chimeric His6CdBP4GFP as represented in Fig. 4*A*. For comparison, the His6GFP was applied as control and exhibited similar results. The other saturated lipids (DPPS, DPPG and DPPA) exhibited the same binding activity. Binding of only minor amounts (5–10%) as compared to the remaining protein in the supernatant of either His6CdBP4GFP or His6GFP to the vesicles was observed (*data not shown*). However, the chimeric His6CdBP4GFP seemed to have more affinity for the liposomes than the His6GFP, especially in the case of DOPC vesicles (Fig. 4*B*). These findings give clear evidence for a preferential binding of the His6CdBP4GFP to fluid-phase lipids.

Effect of Chimeric GFPs on the Isotherm and Interfacial Pressure of Phospholipid Monolayers

To test the binding capacity of the chimeric His6CdBP4GFP to lipid layers at a given interfacial pressure, which is about 30–35 mN/m in the liposome, we investigated the lipid-protein interaction on monolayers at the air/water interface. Figure 5 demonstrates the obtained isotherms of DOPC monolayers before and after injection of chimeric GFPs and the corresponding changes of lateral pressure. Injection of chimeric His6CdBP4GFP underneath a DOPC monolayer at 10 mN/m caused an increase in fluidity and expansion of the surface area of lipid molecule (Fig. 5*A*). An increase in fluidity and surface expansion was also observed upon addition of His6GFP, but much less pronounced (Fig. 5*B*). At high pressure (~40 mN/m), the surface area per lipid molecule before and after protein injection was the same in both cases. This clearly indicates that the chimeric GFP is squeezed out from the lipid monolayer under compression without loss of lipid molecules. Addition of His6CdBP4GFP to DOPC-monolayers at 10 mN/m caused a dramatic increase in the interfacial pressure up to 6.5 mN/m within one hour; a much slower and less pronounced increase of lateral pressure by approximately 4 mN/m was observed in the case of His6GFP (Fig. 5*C*).

The effect of chimeric GFPs on the monolayer isotherms was also investigated in saturated phospholipids, e.g., DPPC. As shown in Fig. 6*A–B*, the surface pressure/area isotherm of DPPC exhibited the typical phase transition at approximately 5 mN/m from the liquid expanded (*le*) to the liquid condensed (*lc*) phase. In the presence of the chimeric His6CdBP4GFP, the isotherm was shifted to a higher area per lipid molecule at low surface pressure, which clearly demonstrates that the incorporation of the chimeric protein caused an expansion of the lipid monolayer. Upon compression, the area per molecule became identical to that of a pure DPPC monolayer at high surface pressure (Fig. 6*A*). Again, an increase in fluidity in the phospholipid phase-transition region was observed upon injection of His6GFP (Fig. 6*B*). However, the fluidization effect was much less compared to the His6CdBP4GFP. A similar pattern upon high compression was revealed.

For a better understanding of the interaction between the His6CdBP4GFP and the lipid mono-

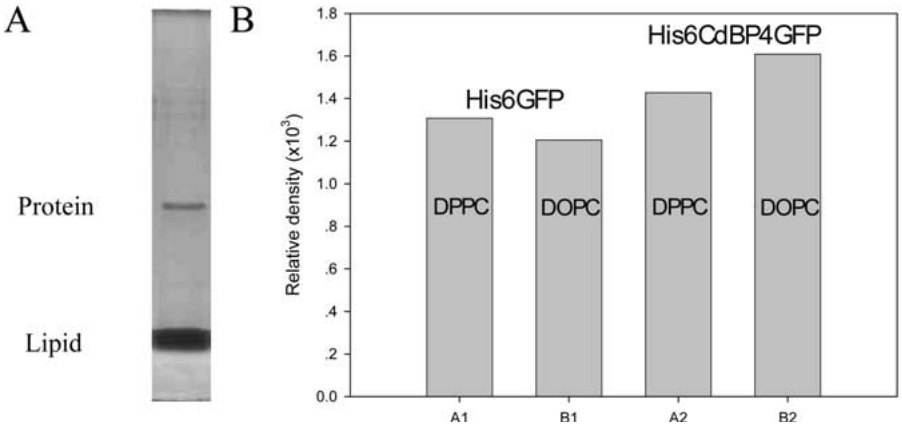

Fig. 4. (*A*) Analysis of lipid-binding capability of chimeric GFP on SDS-PAGE. (*B*) Amount of chimeric His6GFP (*1*) or His6CdBP4GFP (*2*) bound to DPPC (*A*) or DOPC (*B*), as determined by densitometry using Quantity One version 4.2 (Bio-Rad^{TM}).

layer, we injected the chimeric His6CdBP4GFP underneath the DPPC monolayer at constant area and at the initial surface pressure of 10 mN/m. This pressure was chosen to represent the situation at the *le–lc*-transition region (10 mN/m). The increase of surface pressure with time caused by the injection of His6CdBP4GFP was more pronounced than that of the His6GFP (Fig. 6*C*). It is noteworthy that injection of the His6CdBP4GFP caused a two-step (biphasic) increase of the lateral pressure. In the first step the pressure rapidly increased by about 2 mN/m within 5 min, followed by a gradual increase until saturation is reached within 30–45 min. In contrast, the presence of His6GFP caused the change of pressure with a slower rate only and the second step of incorporation was not observed.

To investigate the interaction with a rigidified membrane the chimeric His6CdBP4GFP was injected under the DPPC monolayer precompressed to 25 mN/m, which is above the plateau region. We found that the chimeric protein at this pressure did not affect the physical state of the monolayers (*data not shown*). This infers that the chimeric His6CdBP4GFP is unable to incorporate into the high-pressure lipid layers—an evidence that is also supported by the low binding of His6CdBP4GFP to the liposome.

Epifluorescence Measurements of Interaction between Chimeric GFPs and Lipid Monolayers

Since binding of the chimeric His6CdBP4GFP was restricted to fluid-phase lipids, we applied epifluorescence measurements to DPPC monolayers in the *le–lc* phase-transition region where rigid and fluid domains coexist. Epifluorescence of DPPC monolayers in the presence of His6CdBP4GFP after compression to 10 or 35 mN/m were determined and compared to the effect of His6GFP. As shown in Fig. 7*A*, the His6CdBP4GFP was able to bind to the extended areas of fluid phase (arrow *b*), but binding was much more pronounced to the narrow defect parts (rim) of the rigid domains (arrow *c*). At high compression (35 mN/m), the fluorescence emission of chimeric protein became more condensed. This indicates the enrichment of proteins in the fluid phase (Fig. 7*B*). In the case of His6GFP, only low fluorescence intensity was detected in the DPPC monolayers. A faint fluorescence of fluid phase is observable between the rigid domains (Fig. 7*C*, arrow *a*) and at the defect part up to 10 mN/m (Fig. 7*C*, arrow *c*) but at a very low intensity compared to the fluorescence pattern obtained with His6CdBP4GFP. When the pressure was increased to 20 or 35 mN/m, no fluorescence could be observed.

Discussion

Chimeric GFPs are widely used to tag proteins, but have not yet been systematically investigated with respect to changes in their interaction properties with other cellular compounds. We have used a set of metal-binding chimeric peptides and investigated their interaction with membrane. Modification of GFP even at the extra-chromophore region of the molecule may affect the fluorescence emission intensity, which depends on the nature of the partner peptide and arrangement of the chimeric molecule. From our findings, the presence of the Cd-binding peptide was proven to cause changes in fluorescence emission activity of engineered cells with the difference in fluorescence intensity of engineered cells in the order of His6GFP >>> native GFP > CdBP4GFP $\sim$ His6CdBP4GFP (Fig. 2*A*). At the protein level, the fluorescence intensity decreased in the order His6GFP > CdBP4GFP > His6CdBP4GFP > native GFP (Fig. 3*B*).

The peptide fused to the GFP subsequently affects the localization of the modified GFP molecule intracellularly. We found that the chimeric Cd-binding green fluorescent proteins (CdBP4GFP and His6CdBP4GFP) remained in the cell debris after disintegration of the cell structure. Localization of the chimeric CdBPGFPs in membrane debris as com-

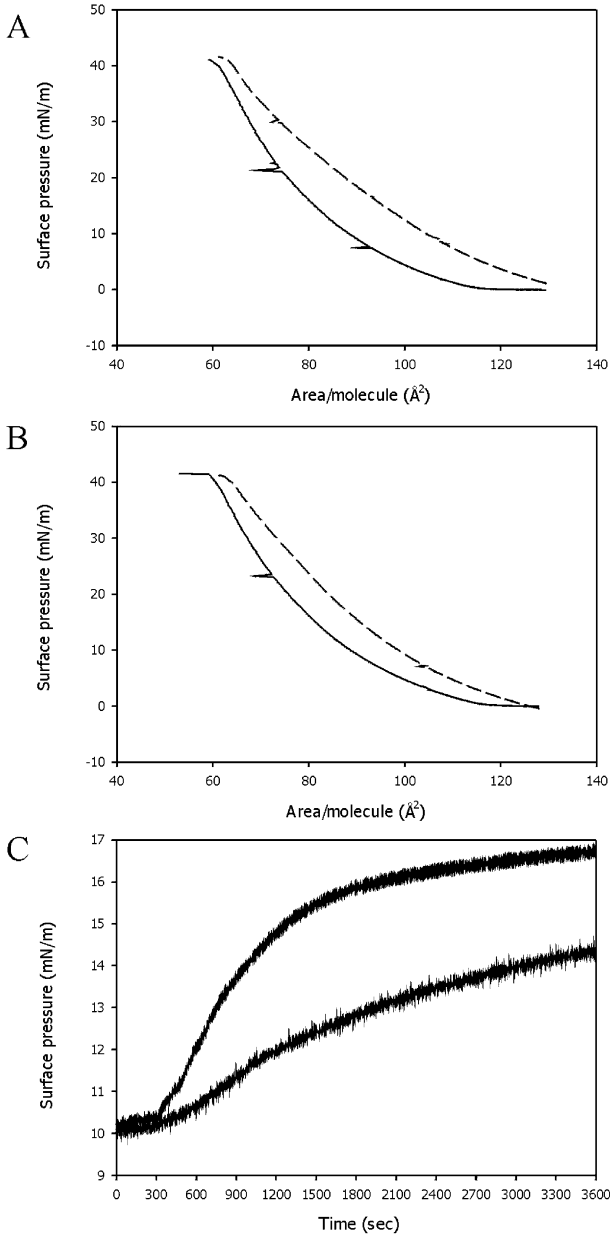

Fig. 5. (*A, B*) Isotherms of DOPC monolayer before (*solid line*) and after (*dashed line*) addition of His6CdBP4GFP (*A*) or His6GFP (*B*). (*C*) Changes of lateral pressure upon injection of 18 nM His6CdBP4GFP or His6GFP underneath the DOPC monolayer.

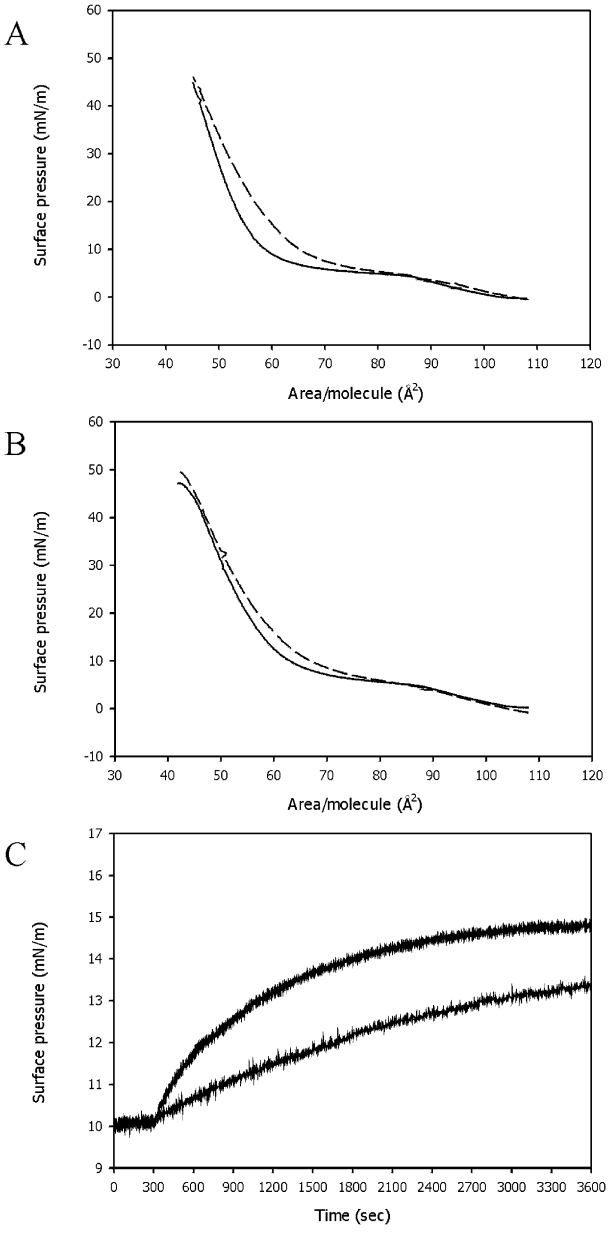

Fig. 6. (*A, B*) Isotherms of DPPC monolayer before (*solid line*) and after (*dashed line*) addition of His6CdBP4GFP (*A*) or His6GFP (*B*). (*C*) Changes of lateral pressure upon injection of 12 nM His6CdBP4GFP or His6GFP underneath the DPPC monolayer.

pared to the other cellular compartments could be detected by the autofluorescence property (Fig. 2*B*). This observation was also reported by others. Cha et al. reported the expression and purification of human interleukin-2 (hIL-2) in insect cells and *E. coli* (Cha et al., 1999a, 1999b; Cha et al., 2000). Fusion of hIL-2 to the GFP caused a localization of the fusion protein in the pellet after cell lysis. Expression of the native GFPuv yielded over 70% of the protein in the soluble fraction. Expression of a fusion protein with hIL-2 exhibited only 13–30% soluble protein. Interestingly, insoluble GFPuv was typically non-fluorescent, so it might be that the fusion protein was soluble but embedded in the membranous material. Similarly, when the hIL-2-GFP was expressed in *E. coli*, the GFP fluorescence was found to be significantly reduced and almost all of the fusion protein was retained in the cell pellet.

Recovery of the chimeric CdBP4GFP and His6CdBP4GFP from the cell debris required 6 M guanidine hydrochloride to solubilize. Neither addition of mild detergent (e.g. Triton X-100) nor changes of ionic strength of the buffer solution caused any

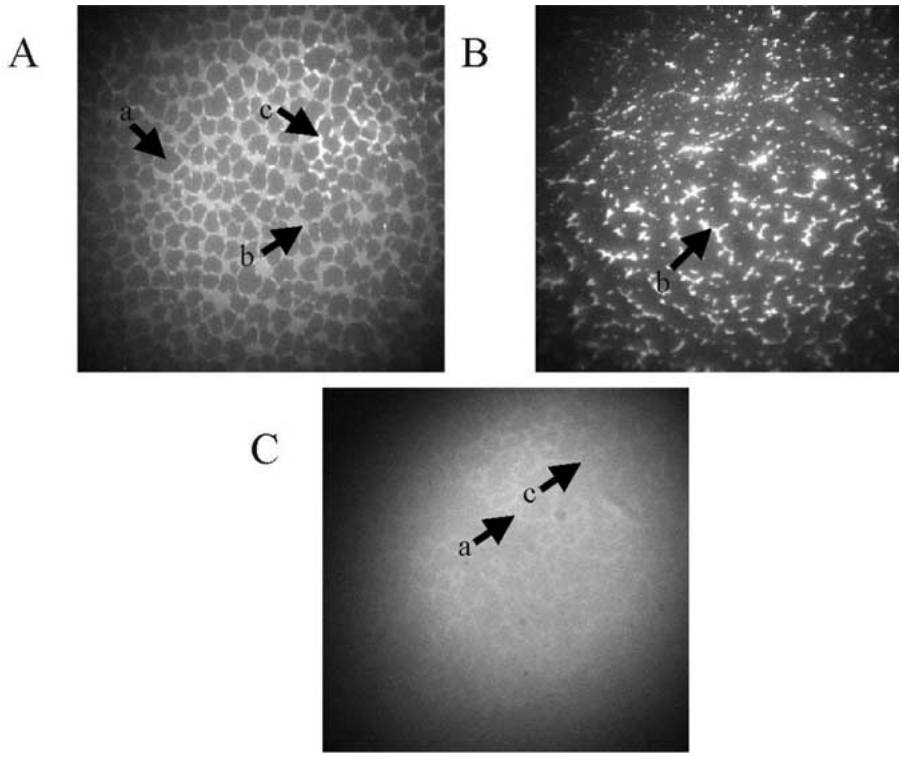

Fig. 7. Epifluorescence of DPPC monolayer in the presence of His6CdBP4GFP (*A, B*) or His6GFP (*C*) after compression at 10 mN/m (*A, C*) or 35 mN/m (*B*) (Subphase: 50 mM Na_2HPO_4, 0.3 M NaCl, pH 7.4 at 20°C). Arrows *a*, *b* and *c* indicate solid domain, liquid domain, and defect part of solid domain, respectively.

expelling effect (*unpublished data*). This again indicates the strong interaction between the chimeric CdBPGFPs with the membrane debris. The possible explanation for this strong interaction might be due to the composition of the Cd-binding peptide. The chimeric CdBP4GFP and His6CdBP4GFP possess tandem repeats of a His-Ser-Gln-Lys-Val-Phe sequence, which contains hydrophobic and hydrophilic amino acids and which are able to bind Cd^{2+}. Such a peptide may undergo conformational arrangements to transfer the chimeric GFP into a more lipid-soluble form, thus causing membrane association. This was strongly supported by the earlier evidence for a decrease of lactate dehydrogenase activity in the cytosol as compared to the total protein of the cell with increasing number of tandem sequences of the Cd-binding peptide to the chimeric LDH (Isarankura Na Ayudhya, 2000). Similar effects of amino-acid composition on the binding properties of the GFP to lipid membranes has also been observed when the protein was fused to natural membrane-bound peptides. For example, the effect on caveolae, the vesicular invaginations of the plasma membrane, has been studied using GFP-caveolin constructs. A short membrane-attachment sequence (KYWFYR) within the caveolin-1 has been fused to the GFP. These six residues, which consisted of the central aromatic and flanking basic residues, were required for membrane attachment. This sequence was sufficient to anchor the soluble cytoplasmic GFP to membranes. Removal of this sequence prevented membrane attachment in cells. In addition, the lack of the two basic amino acids (lysine and arginine) prevented the adequate localization of GFP in the membrane pellet. These results suggested the need for electrostatic interactions mediated by the flanking basic residues in addition to the hydrophobic interaction caused by the aromatic residues for membrane association (Woodman et al., 2002).

To further investigate membrane-binding properties of the chimeric green fluorescent proteins, here the binding of the chimeric His6CdBP4GFP to artificial lipid membrane was determined as compared to that of the His6GFP. First focussing on the interaction of chimeric His6CdBP4GFP with lipid vesicles, we observed the binding of a small amount of the His6CdBP4GFP to the lipid (Fig. 4). This might be due to the high surface pressure of lipid bilayer of about 30 mN/m. When the His6CdBP4GFP was injected underneath the lipid monolayer compressed to a variable low initial pressure, a considerable increase of the interfacial pressure was observed. Furthermore, an increase in fluidity corresponding to an expansion of the surface lipid layer was shown (Figs. 5 and 6). At high pressure, the His6CdBP4GFP could not incorporate into the lipid monolayers. This evidence strongly supports our finding of the low binding of protein to lipid vesicles.

Second, the effect of lipid fluidity upon protein binding was also investigated. The chimeric His6CdBP4GFP seemed to have more affinity for unsaturated phospholipids than for saturated lipids (Figs. 4–6). This was supported by the fact that the

His6CdBP4GFP preferentially binds to fluid phase and to the defect parts of lipid domains, as determined by fluorescence microscopy (Fig. 7). The chimeric His6CdBP4GFP exhibited strong fluorescence upon incorporation to the liquid phase of a DPPC-monolayer, while very low intensity could be observed in the case of His6GFP. A possible explanation might be non-specific adsorption of His6GFP to the lipid, which caused unfolding of the protein accompanied by loss of fluorescence within a few seconds. This result was also reported by Dorn et al., namely, that the fluorescence of GFP coupled to hexahistidine vanished within 20 min, whereas the lateral pressure was not decreased (Dorn et al., 1998). Third, the interaction of His6CdBP4GFP with DPPC-monolayers exhibited biphasic kinetics, including a rapid initial phase and a slower second phase (Fig. 6*C*). In contrast, the His6GFP induced only the first phase, however, with a slower rate of pressure increase compared to the His6CdBP4GFP. The lack of the second phase indicated that the pressure increase within this phase might be due at least partially to the specific insertion of the Cd-binding peptide into the monolayer. In contrast to the second-phase interaction, the first phase was due to non-specific interactions between an integral part of GFP and the monolayers, as evidenced by the fact that the His6GFP was capable of inducing this rapid phase to a lesser extent than the His6CdBP4GFP (Fig. 6*C*). However, the effect of various concentrations of these chimeric GFPs on changes of lateral pressure need to be further investigated. Determination of binding constants between these chimeric GFPs and lipid molecules has to be envisaged. Fourth, we demonstrated that the chimeric His6CdBP4GFP was bound peripherally to the lipid monolayers. Upon injection of the chimeric protein underneath the lipid monolayer, the isotherm was shifted to higher area per lipid molecule at low surface pressure. Upon compression to high pressure, the area per molecule became identical to that of a pure DPPC-monolayer (Figs. 5 and 6). This indicates that the chimeric GFP is squeezed out of the lipid monolayer, in agreement with our fluorescence microscopy data. At high pressure (35 mN/m), the fluorescence became more condensed, caused by the reduction of the fluid-domain area. At 50 mN/m, very low fluorescence intensity could be observed due to the rigid packing of the lipid monolayer (*data not shown*). All these findings indicate the higher binding affinity of the His6CdBP4GFP to fluid phase domains within lipid monolayers as compared to His6GFP.

Two aspects seem to be relevant for discussion with respect to the use of GFP-constructs in general. First, changes of hydrophobicity of a protein upon insertion of the fusion partner and consequent interaction at the cellular level have to be considered. Second, the effects of the fusion partner on the lipid-binding properties of the construct opens up more useful applications in cell biology and biotechnology. For example, the GFP tagged with a cysteine-rich domain from protein kinase C (Cys1-GFP) was constructed and applied as fluorescence indicator for diacylglycerol signaling in mammalian cells. The cysteine-rich domain provided affinity not only for zinc ions but also for lipid membrane in the presence of diacylglycerol or phorbol ester. Therefore, transient translocation of cytosolic Cys1-GFP to the plasma membrane was observed upon stimulation of G proteins or tyrosine kinase-coupled receptors (Oancea et al., 1998; Wang et al., 1999; Hurley & Meyer, 2001). Moreover, the insulin receptor substrate (IRS) protein and the pleckstrin homology domains (PH) were fused to the GFP and applied as reporters for subcellular localization. These chimeric proteins were found to be localized exclusively in the cytoplasm. Stimulation with insulin caused a translocation of the chimeric protein to the plasma membrane within 3–5 min (Hurley & Meyer, 2001; Razzini et al., 2000). Moreover, Obrdlik et al. (Obrdlik, Neuhaus & Merkle, 2000) constructed and expressed a chimeric protein between β-subunit of G-protein and GFP in transgenic plants. They demonstrated that G_β was located at the membrane surface and attached to membranes via hydrophobic interactions. Mutation in the β-domain caused severe decrease of the membrane association.

Beyond those useful applications, we have previously applied all these chimeric GFPs and engineered cells as a potential tool for metal determination (Isarankura Na Ayudhya, 2000; Prachayasittikul et al., 2000, 2001). Therefore, this study opens up the possibility to attach the chimeric metal-binding GFPs onto membrane surfaces while they may then be applied for development of a fluorescent membrane-based metal sensor or a biofunctionalized membrane in the future, e.g., as a supporting layer on a glass fiber (Klee et al., 1995; Dietrich et al., 1996; Nock, Spudich & Wagner, 1997; Tvarozek et al., 1998; Kostov, Albano & Rao, 2000).

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