The Spatial and Temporal Dynamics of Pleckstrin Homology Domain Binding at the Plasma Membrane Measured by Imaging Single Molecules in Live Mouse Myoblasts*

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Pleckstrin homology (PH) domains act to target proteins to the plasma membrane and intracellular vesicles by binding to specific phosphoinositol phospholipids. We have investigated the binding kinetics of PH domains found in the tail region of the molecular motor, myosin X. Using total internal reflection fluorescence microscopy, we observed binding and release of individual PH domains fused to green fluorescent protein at the plasma membrane of living cells. Individual spots of light corresponding to single fluorescently tagged molecules were imaged onto a sensitive camera system, and digital image processing was then used to identify each fluorophore and store its trajectory in time and space. The PH domains bound with an apparent on-rate of 0.03 µm⁻¹ µm⁻² s⁻¹ and a detachment rate constant of 0.05 s⁻¹. The average residency time of the domains at the plasma membrane was about 20 s. We found very limited movement of the membrane-bound PH domains in the mouse myoblast cells that we studied. This implies that the PH domains must either be attached to the cytoskeleton or corralled in a lipid compartment. Localization of the PH domains together with their rapid detachment rate is probably important in controlling the response of myosin X to signaling events and in regulating its cellular function.

PH domains are found in over 250 proteins in the human genome (1) and are known to bind specific phosphoinositol phospholipids (PtdIns). The binding of PH domains to their target phospholipids is controlled by phosphorylation of the inositol moieties of the lipids. Because there are five available phosphorylation positions, this could give rise to 32 different PtdIns forms (for example, a 5-bit binary encoding). Although binding affinities of different PH domains have been measured in vitro and their localization has been observed in vivo, little is known regarding the membrane binding dynamics of these domains. Recently, several laboratories have developed optical methods to make wide field observations of individual fluorophores in aqueous solutions and these techniques enable the dynamic behavior (e.g. kinetics of membrane binding and release, diffusion times, and trafficking) of proteins, ligands, and viruses (2–7) to be measured simply by making steady-state observations. However, one of the main problems in imaging single fluorophores within a living cell is the high autofluorescence that overlaps the emission spectrum of the enhanced green fluorescent protein (eGFP) used in many such studies (8). To overcome this problem, we exploited total internal reflection fluorescence microscopy (TIRFM) to observe single fluorophores in living cells. TIRFM is a wide-field method (e.g. illuminates the entire cross-section of a specimen) that relies on near-field excitation (by the evanescent wave); thus, only a thin layer of the cell that lies close to the glass-water interface is illuminated (9). This technique enables the activity of individual molecules to be observed directly allowing membrane binding dynamics of single molecules to be measured by steady-state observation.

We are interested in the behavior of myosin X, which is a dimeric molecular motor consisting of a highly conserved motor domain that interacts with actin to produce force and movement and a modular tail region that is thought to bind to membranes via its PH domains. Here, we studied the three tandem PH domains present in its tail region (10). The first domain is split, such that the second domain is inserted into a loop in the first domain. The tail domain of myosin X fused to eGFP localizes to the actin rich cortex (11), and of the three identified tail domains, only the PH domains show any membrane localization (12). Sequence homology mapping suggests that all three domains bind to phosphoinositol lipids with a phosphate at the 3’ position such as PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ following phosphatidylinositol 3-kinase phosphorylation of PtdIns(4,5)P₂ upon cell stimulation (13). Phospholipid blot overlay assays showed that the PH domains in fact bind to PtdIns(3,4,5)P₃, PtdIns(3,4)P₂, and with lower affinity to PtdIns(4,5)P₂ in vitro. In vivo, the PH domains are recruited to the plasma membrane, areas of dynamic ruffling activity, and intracellular vesicles in live cells.

In this study, the binding kinetics of single molecules of eGFP-PH123 at the plasma membrane of live cells was investigated using TIRFM. Time-lapse TIRFM was employed to visualize individual fluorophores for long periods (>500 s), and this enabled data from up to 1000 individual fluorophores to be collected from each cell specimen. By handling the data obtained from each individual molecule separately, the power of the single molecule approach is retained, whereas sufficiently

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§ The abbreviations used are: PtdIns, phosphatidylinositol; PH, pleckstrin homology; TIRFM, total internal reflection fluorescence microscopy; PtdIns(3,4,5)P₃, phosphatidylinositol 3,4,5-triphosphate; PtdIns(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,5)P₂, phosphatidylinositol 3,5-bisphosphate.

1 The average residency time of the domains at the plasma membrane was about 20 s. We found very limited movement of the membrane-bound PH domains in the mouse myoblast cells that we studied. This implies that the PH domains must either be attached to the cytoskeleton or corralled in a lipid compartment. Localization of the PH domains together with their rapid detachment rate is probably important in controlling the response of myosin X to signaling events and in regulating its cellular function.

2 D. Tacon and M. Peckham, unpublished findings.

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large data sets could be collected to ensure that the results are statistically valid. Steady-state observation of single molecules enabled their binding kinetics and spatial dynamics to be measured without perturbing the cell. Furthermore, the use of a novel time-lapse illumination method to systematically change the illumination intensity enabled the effects of photo-bleaching to be obviated.

**EXPERIMENTAL PROCEDURES**

Cloning—The full-length myosin X cDNA was amplified from mouse myoblasts by long reverse transcription-PCR using primers based on the mouse sequence (GenBank accession number AJ249708) (14). It was cloned into pUC19 in its epitope to check for sequence fidelity. A fragment containing all three PH domains (eGFP-PH123, nucleotides 3615–4500) was subcloned into either pEGFPN1 or pEGFPCl (Clontech), such that eGFP was fused either to the N terminus with a 15 amino acid linker or the C terminus with a 6 amino acid linker. In the experiments reported here, we used exclusively the C-terminal constructs. These were transiently expressed in COS-7 cells, and the cell lysates were immunoblotted using an anti-GFP antibody (AbCam Ltd., Cambridge, United Kingdom) to confirm that the expressed proteins were of the predicted size (data not shown). In the experiments described here, we used eGFP-PH123 in which eGFP was fused to the C terminus; however, we found that N-terminal fusions gave similar cell localization.

Cell Culture, Transfection, and Expression Levels—Mouse myoblasts isolated from the “immortal mouse” (Htk-/-A58) were cultured as described previously (15). They were transfected with the eGFP constructs using FuGENE 6 (Roche Diagnostics) or Genejuice (Novagen) following the recommended protocol. After 24 h, the cells were plated onto glass coverslips and allowed to settle. 8 h later, the medium was replaced with Hanks balanced salt solution containing 20 mm HEPES (pH 7.4) without serum and the coverslip was assembled into a small chamber for viewing the cells on the inverted microscope (see below). Approximately, 10% of the cells had a suitable level of expression for TIRF measurements following this transfection procedure, such that single molecules could be visualized as single spots.

The final concentration of eGFP fusion protein in cells used in the TIRF experiments was in the nanomolar range (i.e. ~1 molecule/μm^2, 5000 molecules/cell). This was confirmed by independent measurements using confocal microscopy where fluorescence of a range of concentrations of eGFP was compared with that of live transfected cells. From the calibration curve, we estimated the concentration to be around 10 nM (9). Under equivalent conditions in neutrophils, the concentration of target lipid, PtdIns(3,4,5)P_3, in the plasma membrane was ~50 nM (local concentration of 5 μM) (16).

Objective Type TIRF Microscopy—The imaging apparatus was based around an inverted microscope (Axiovert S100, Zeiss) with a high numerical aperture objective lens (Fluor 100, 1.45NA, Zeiss). An argon ion laser (532-AP-A01, Melles Griot) was used to illuminate the cells using a custom optical arrangement to produce evanescent field excitation (TIRFM) (9). Illumination was controlled using a mechanical shutter system and neutral density filter. A small (~2.5 mm diameter) mirror placed close to the back aperture of the objective lens directed the laser beam to the periphery of the lens so that incident light approached the specimen at an angle exceeding the critical angle for total internal reflection. A second mirror on the opposite side of the back aperture reflected the totally internally reflected beam out of the microscope system and projected it onto a position-sensitive photodiode used for autofocus control (see Ref. 9 for a full description). Sequences of images were acquired by a digital image-intensified CCD camera (GemStar, Photonic Science, Robertsbridge, United Kingdom) and recorded to the hard drive of a PC for analysis.

Microscope Observation Chambers—Cells transfected with eGFP-tagged PH domains were plated onto glass coverslips and mounted in a viewing chamber. The chamber consisted of a Perspex block with a coverslip base. The Perspex block with adhered coverslip was fixed to the lower face of the Perspex block, and the central chamber was then quickly filled with medium to prevent the sample from drying out. The upper face was then covered with a second coverslip to seal the central chamber. Small sideports enabled bathing media to be exchanged.

**Visualizing eGFP Molecules in Vitro Single Fluorescence-imaging System**—To calibrate the single fluorophore TIRFM system, purified eGFP was viewed under controlled conditions. Microscope flow-cells were constructed using a pre-cleaned 22 × 50-mm^2 glass microscope slide which across two 3 × 22-mm² strips of (number 1) coverslip were fixed 15 mm apart using 2 μl of UV-curing epoxy adhesive (RS Components, Corby, United Kingdom). A 22 × 40-mm² pre-cleaned coverslip was glued onto the coverslip strips orthogonally to the slide, leaving ~10 mm of coverslip projecting from either side. The flow-cell was exposed to UV light until the glue was completely cured. 50 μl of a buffered solution (pH 7.4) containing 5 μg ml^{-1} anti-eGFP antibodies (Abcam Ltd) was applied to the flow-cell for 5 min. This was subsequently washed out, and the surface was blocked using 1 mg ml^{-1} bovine serum albumin. 50 μl of a buffered solution containing 20 ng ml^{-1} eGFP (Clontech) then was introduced for 5 min, and finally, unbound eGFP was washed out using an excess of buffer solution. Flow-cells were then viewed using the TIRFM microscope. The eGFP attached to the glass surface at a density of 0.2–0.3 molecule μm^{-2}. Single eGFP molecules were clearly seen on glass surface as isolated spots of light. In this size, intensity, and lifetime, individual fluorophores together with their characteristic rapid photo-bleaching were used to calibrate the system for later studies using live cells. Live cell studies were conducted using the same microscope, and camera settings were used as the control studies. All of the experiments were conducted at 35 °C.

**Identification of Single Fluorophores, the “DISH Criteria”—** Image data was analyzed using a custom written single fluorophore detection algorithm (9). Control data sets obtained from eGFP molecules attached to the coverslip via antibody showed properties characteristic of single fluorophores. We term these the DISH criteria whereby the fluorescent spots of light had 1) diffusion-limited size, 2) intensity of emission characteristic for a single fluorophore, 3) single step photobleaching, and 4) half-life of the fluorescent population before photobleaching occurred, directly proportional to laser excitation power.

Based on these criteria, we developed a three-pass computer algorithm that performed spatial and temporal statistical tests on stored video sequences to identify individual fluorophores (see below). On the first pass, groups of pixels were analyzed in time to test for the expected sudden change in intensity characteristic of rapid appearance and disappearance of single fluorophores. Fluorescent spots that exhibited the correct amplitude and speed of intensity change and which also had the correct diffusion-limited size were then subjected to a second round of analysis. The second pass performed a statistical analysis of the intensity levels before and after candidate changes in intensity were noted during the first pass. On the final pass of the algorithm, the trajectory in space and time for each candidate fluorophore was determined by tracking the centroid of each fluorescent spot. The computer program produced an output file that could be checked against the original raw data sets and which also contained all of the statistical information regarding the individual time trajectories for each fluorophore. The global statistics concerning average lifetimes, fluorescence images produced using TIRFM were typified by the fact that fluorophores diffuse very rapidly to and from the evanescent excitation region before and after binding at the plasma membrane (which is close to the coverslip interface). This means that spots of light appear rapidly and then persist at the membrane for several video frames until they either photobleach or the molecule detaches. In either case, the spot of light rapidly disappears. The time resolution of our system is insufficient to discriminate between unbinding followed by rapid diffusion out of the excitation field and instantaneous photobleaching. In fact, the average diffusion time for a small protein in cytoplasm (e.g. diffusion coefficient, D = 1 × 10^{-9} m² s^{-1}) from an evanescent field of depth, x = 100 nm, is given by τ = (2Dx)^{1/2}.

Because photobleaching is directly proportional to laser power, whereas unbinding is independent of illumination power, photobleaching can be factored out from unbinding by systematically changing the average laser power. To accomplish this, time-lapse illumination was employed. In every 5-s interval, the laser was switched on for 0.33, 0.55, 1.05, or 1.55 s or left on continuously for the full 5 s. The illumination “duty cycle ratio” was varied over the range from ~7 to 100%. This approach offers the advantage that signal-to-noise ratio of the camera system and the TIRF angle during the period of illumination remain constant.

**RESULTS**

**Imaging of Single eGFP Molecules in Vitro—** Purified eGFP attached to coverslips via anti-GFP antibodies could be identified clearly as isolated diffusion-limited spots of light (Fig. 1a). The spatial intensity distribution produced by individual fluorophores should correspond to an Airy disc pattern with a characteristic point spread function determined by the imaging system. Full width at half-height should be close to 0.6λ/NA.
Fig. 1. Purified eGFP (Clontech) was bound to antibody (Abcam Ltd) that had been adsorbed to a cleaned glass surface. Single fluorophores appear as individual spots of light. (a) Wide-field view of eGFP stuck to antibody. Inset, plot of intensity profile for one spot (width at half-height is 400 nm limited by the resolution of the imaging system). (b) Intensity versus time trajectory for one spot. Inset, histogram of intensity including bright and dark periods and dark counts produced a peak at low values, and the fluorophore produced a signal with mean of 80 units ± S.D. of 20. The noise is due to photon-counting statistics (80 camera counts = 30 photons). (c) Intensity distribution of mean intensities of all spots. Note the broad distribution in intensities (much broader than expected from photon noise in (b)) due to other sources of variance. (d) Distribution of times to photobleaching, average lifetime here was 3.5 s. The average time to photobleaching derived from such a plot is inversely proportional to the bulk photobleaching rate measured for an ensemble. A.U. arbitrary units.

250 nm (where \( \lambda \) = optical wavelength, N.A. = effective numerical aperture of the imaging system, maximum of 1.3 for aqueous specimens). Our system gave a measured width at half-height of 400 nm (Fig. 1a, inset). This was dominated by image degradation caused by the image-intensifier system.

Plots of intensity levels of individual fluorophores against time showed an approximately constant level of fluorescence with noise level dominated by photon counting (Fig. 1b). Approximately 30 photons/image frame were collected so that the expected mean ± S.D. in intensity due to photon counting statistics was \( 30 \pm 5 \) (i.e., 20%) of the mean intensity (Fig. 1b, inset). The distribution of mean intensities measured for many different fluorophores (Fig. 1c) is much broader than expected from the noise content of an individual record (e.g., Fig. 1b indicates that the average intensities should be within 1 count). The large variation in individual intensities probably arises from the random orientation of the eGFP on the surface (because polarized light was used to excite fluorescence) and variations in its local environment. Histograms in which the distribution of lifetimes of individual fluorophores before photobleaching were plotted show that the rate of disappearance of fluorophores due to photobleaching was monoexponential (Fig. 1d) with a half-time that depended in a linear fashion on the illumination intensity (see Fig. 4).

**Imaging Single eGFP-PH123 Fluorophores in Live Cells—**

TIRFM was used to image eGFP-PH123 expressed in living cells at very low expression levels (<10 nM) (Fig. 2a). Individual spots of light were observed (Fig. 2b) that had the same statistical properties as control experiments made using purified eGFP bound to a glass surface via antibody (see “Experimental Procedures”). When the cells were continuously illuminated at high laser power, the average fluorescence of the whole cell decreased (Fig. 2c). The exponential time course of bulk photobleaching was similar to the average lifetime of the individual fluorescent spots (\( \tau = 2.5 \) s in Fig. 2, d and e). The conclusion from this finding is that most of the observed fluorophores are probably being photobleached during the observation period.

When the average illumination intensity was decreased so that the cells were illuminated for only 350 ms in any 5-s period, the overall fluorescence intensity of the cell then remained high for many hundreds of seconds (Fig. 3, a and d) and decreased by only 15% over the entire recording period (900 s). Under these conditions, fluorescent spots arrived and remained at the membrane for many seconds (Fig. 3, b and c), the average lifetime of the spots was now 14 s (Fig. 3c), and some lasted as long as hundreds of seconds. eGFP-PH123 that detached from the membrane before photobleaching diffused from the excitation field so rapidly (<0.5 ms) that our camera system could not directly distinguish this from instantaneous photobleaching.

Illumination power was varied systematically as before (using purified eGFP bound to an antibody-coated coverslip), and the average lifetime of the fluorophores before photobleaching was found to depend linearly upon illumination power. However, a plot of the rate constant derived for the disappearance of the spots (\( k_{\text{off}} \) = inverse of the half-life) versus average laser power (Fig. 4) now showed a non-zero intercept. \( k_{\text{off}} \) should be the sum of two rate processes, photobleaching and detachment (\( k_{\text{ph}} \) and \( k_{\text{d}} \), respectively). Photobleaching rate, \( k_{\text{ph}} \), will be proportional to illumination power, \( \xi \), and assuming that these
processes are irreversible, \( k_{\text{off}} = \xi \times k_{\text{ph}} + k_d \). Thus, at high average laser power, the dominant process will be \( \xi \times k_{\text{ph}} \), whereas at low power (\( \xi \approx 0 \)), it will be \( k_d \). This analysis for eGFP-PH123 in living cells showed that at zero illumination, \( k_{\text{off}} = k_d = 0.05 \text{ s}^{-1} \) (Fig. 4). In contrast, eGFP molecules attached via antibodies to glass had an extremely slow detachment rate, \( k_d \), and, therefore, \( k_{\text{off}} \) extrapolated to near zero at zero laser power (Fig. 4). Our analysis assumes a single exponential (i.e. simple Poisson) process for both photobleaching and unbinding. The error in estimating the mean lifetime depends upon: 1) the sampling statistics; 2) systematic noise; and 3) whether or not the underlying model is actually suitable and correct. In the figure legends, we give the \( R^2 \) value to indicate the goodness-of-fit of the monoexponential model to the histogram bins. The \( R^2 \) values indicate that a single exponential is an adequate description of our data. Because our sample size is \( \sim 500 \) observations for most experiments, as a “rule-of-thumb,” the 95% confidence interval would be \( \sim \)10% of the mean as shown in Equation 1,

\[
\text{95\% confidence interval} = 2 \text{ S.D.} = \frac{2\mu}{\sqrt{n}} \quad \text{(Eq. 1)}
\]

where \( \mu \) = mean lifetime, \( n \) = number of samples, and S.D. = mean \( \pm \) standard deviation.

The apparent binding rate of eGFP-PH123 at the plasma membrane was estimated by analyzing the “landing rate” (i.e. the distribution of dark intervals before a fluorescent spot appeared within a given area of membrane or sample quadrat). Landing rate depended in a linear way upon the observation window bin size (i.e. area of the sample quadrat). Best estimates of landing rate were obtained using a 5 \( \times \) 5-\( \mu \text{m}^2 \) quadrat. We found that the apparent binding rate, \( k_{\text{on}} \), of eGFP-PH123 at the plasma membrane was \( 0.28 \times 10^{-3} \text{ \mu m}^{-2} \text{s}^{-1} \) (Fig. 5). The apparent binding rate, \( k_{\text{on}} \), depends upon the concentration of free eGFP-PH123 in the cell, the density of binding sites (with the correct phosphoinositol moiety), and the concentration of any competing PH domains. The expression level of eGFP-PH123 was found to be \( \sim \)10 nM by comparing the fluorescence signal measured in the cytosol of cells to that of free eGFP in solution by confocal microscopy (9). Using this estimate of the concentration of free eGFP-PH123, the apparent second order rate constant for binding, \( k_{\text{on}} \), is calculated as \( 0.028 \text{ \mu m}^{-1} \text{\mu m}^{-2} \text{s}^{-1} \).
FIG. 3. Detection of single molecules of eGFP-PH123 molecules in the lamella of a living mouse myoblast under time-lapse recording. An example of one cell that was illuminated for 350 ms in every 5-s interval, giving an illumination duty ratio of 0.07. a, individual TIRF images taken at 0, 8, and 15 min during a time-lapse recording. The majority of the fluorescent spots appeared on the membrane after the beginning of the record. b, four representative fluorescence intensity tracks of the diffraction-limited areas (5 × 5 pixels, 0.16 μm²) for single fluorophores detected during the record in a. c, image sequence of a single fluorophore that landed on the cell membrane and stayed attached for over 140 s together with the fluorescence intensity histogram of this spot measured at 5-s intervals. d, the average cell fluorescence decreased slightly during recording (by ~15%). e, histogram showing the lifetime distribution of 775 individual spots. The distribution was fitted by a single exponential with a rate constant of 0.07 s⁻¹ (τ = 14s, R² = 0.98). Average laser power density was 10 microwatts μm⁻². A.U., absorbance unit.

FIG. 4. Calculation of eGFP-PH123 domain dissociation rate. Lifetime of single eGFP-PH123 molecules on cell membrane depends on the photobleaching and dissociation rate (k_μ = \xi × k_{off} + k_d). k_{off} was linearly dependent on the average laser illumination, \xi (illumination duty ratio) (open circles). A linear regression fit to the data gives the intercept, k_d, as 0.05 s⁻¹ for eGFP-PH123 (squares). By comparison, eGFP molecules attached to GFP antibodies on the glass surface (circles) show that k_d is close to zero (i.e. eGFP is very tightly bound to antibody, and antibody is tightly bound to the glass). For the eGFP on antibody experiment, the laser power density was decreased from 10 to 3 microwatts μm⁻² to obtain a similar fluorophore lifetime distribution to eGFP-PH123 in cells.
Direct Observation of Individual PH Domains

The distribution of landing times was plotted as a histogram and fitted to a single exponential process. This gave the probability of binding to a site per unit area per second. To perform this analysis, we actually experimented with several different sized bins but found that when we varied the quadrat size systematically, the estimated landing rate per \( \mu m^{-2} \) was similar, regardless of its size. The observed landing rate here was 0.007 s\(^{-1}\) \( (fitted\ line) \), giving a rate per unit area of 0.28 \( \times 10^{-3} \) \( \mu m^{-2} s^{-1} \).

**BINDING KINETICS FOR eGFP-PH12 AND eGFP-PH3**—The above experiments were repeated using the first two PH domains of myosin X (PH12) and the third domain (PH3) fused to eGFP to determine the relative contribution of each PH domain to the observed binding and release rates at the plasma membrane. Neither of these constructs bound as tightly as eGFP-PH123, and there was a higher proportion of free to bound material. At an illumination duty ratio of 0.07%, \( k_{off} \) was 0.14 s\(^{-1}\) for both eGFP-PH12 ( \( n = 800 \) ) and eGFP-PH3 ( \( n = 300 \) ). From the relationship, \( k_{off} = k_{cat} \times k_{ph} + k_{d} \) and \( k_{d} \) is calculated as 0.12 s\(^{-1}\) (knowing that \( \xi \times k_{ph} \) at 7% power was 0.021 s\(^{-1}\)).

**LATENT MOVEMENT OF PH DOMAINS ATTACHED TO THE PLASMA MEMBRANE**—We tracked the position of fluorophores bound at the plasma membrane by fitting the intensity distribution obtained from the sum of three adjacent (100 ms) frames to a three-dimensional Gaussian mask of variable amplitude and position but of fixed width. We found that the mean-squared deviation in position did not increase with time but instead remained close to the limit of our position detection (0.03 \( \mu m^2 \)). The root-mean-squared deviation (e.g. the amount of movement that one would perceive by eye) was only 170 nm, and video images of the fluorescent spots therefore appeared fixed in position when viewed by eye. For a freely diffusing particle, one would expect the mean-squared displacement in position to increase linearly with time, such that mean-squared displacement = 4D \( t \), where \( t \) = time and D = lateral diffusion coefficient (with units in \( \mu m^2 s^{-1} \)). The extremely limited movement of the PH domains found in these experiments appears to be a feature of the mouse myoblastic cell line with which we chose to work. In other experiments using human umbilical vein endothelial cells, we found that the same fluorescently tagged PH domains (i.e. using identical genetic construct and experimental conditions) diffused haphazardly over many micrometers during a typical observation period of 5–10 s.

**DISCUSSION**

Isolated PH domains have been used previously to determine the cellular localization of lipids such as PtdIns(4,5)P\(_2\), PtdIns(3,4,5)P\(_3\), or PtdIns(3,4)P\(_2\). Here, we have observed individual eGFP-PH123 molecules binding to the inner leaflet of the plasma membrane. Steady-state observations of single fluorophores lead to estimates for both binding and unbinding of these PH domains inside the living cell. All three PH domains present in the tail of myosin X are required for tight binding to membrane. Using a time-lapse method to systematically adjust the laser excitation power, the separate rates of photobleaching and detachment or unbinding of eGFP-PH123 were measured. The off-rate and apparent on-rate for this binding process together with the rate of lateral movement were calculated from the single molecule data sets.

From lipid blots, eGFP-PH123 is expected to bind to PtdIns(4,5)P\(_2\) and PtdIns(3,4,5)P\(_3\) at the plasma membrane. PtdIns(4,5)P\(_2\) predominates in unstimulated cells, whereas levels of PtdIns(3,4,5)P\(_3\), which is produced by rapid bursts of synthesis from PtdIns(4,5)P\(_2\) in response to cell stimulation, are probably low in the serum-free conditions used here. Previous studies (17–20) suggest that PtdIns(4,5)P\(_2\) is localized to discrete regions of the plasma membrane such as lipid rafts, membrane ruffles, and regions of the membrane associated with F-actin (e.g. focal adhesions) (21). For example, it has been suggested that PtdIns(4,5)P\(_2\) acts to restrict actin polymerization to the cortex and PtdIns(3,4,5)P\(_3\) controls the region of cortex at which actin polymerizes (17). Although the TIRFM excitation method used here means that we were unable to observe activities in ruffles, which project above the cell and out of the region of the evanescent wave, the discrete spots that we did observe at the plasma membrane for eGFP-PH123 could potentially correspond to lipid rafts or anchorage sites for F-actin at focal adhesions.

Measurements of the lateral movement of eGFP-PH123 bound to plasma membrane were surprising in that the fluorophores remained fixed (within the resolution of our measurements, e.g. root mean square = 170 nm) in position for the entire observation period. Previous measurements (22–24) of the free diffusion of fluorescently labeled phospholipids range from 0.18 to 5.4 \( \mu m^2 s^{-1} \). The restricted diffusion for PtdIns(3,4,5)P\(_3\), measured using AKT fused to eGFP, was 0.5 \( \mu m^2 s^{-1} \), although single molecules were not imaged in that study (24). The restricted lateral movement observed in the present study might be explained by the binding of PH domains either directly or indirectly to the actin cytoskeleton through a second binding site. Alternatively, PtdIns(3,4,5)P\(_3\) phospholipids might be more diffusible than PtdIns(4,5)P\(_2\) or the lateral diffusion rates might have been overestimated in previous studies.

The limited residency time (≈20 s) of eGFP-PH123 molecules at the cell membrane is similar to previous estimates (24). This is probably important for dynamic rearrangement of the actin-based cytoskeleton during cell movement and during endocytic and exocytic events. PtdIns(4,5)P\(_2\) is linked to endocytic events, and PtdIns(3,4,5)P\(_3\) is linked to exocytosis, such that membrane ruffling results from insertion of vesicles from the endocytic pathway into the plasma membrane (25). Myosin X has been suggested to be important in intrafilopodial motility and phagocytosis (26). The present TIRFM studies show that isolated domains bind tightly at the plasma membrane and probably target myosin X to this location in vivo. The work presented here, together with earlier studies, implies that the PH domains of myosin X probably act to recruit the motor to sites of actin polymerization at the cell cortex. The dissociation rate, \( k_{diss} = 0.05 s^{-1} \), is appropriate for myosin X targeting to respond to changes in levels of PtdIns(3,4,5)P\(_2\) or PtdIns(4,5)P\(_2\) following cell stimulation.

In earlier experiments not reported here, we worked with a...
full-length GFP-tagged myosin X construct that unfortunately contained two random (PCR) mutations within the myosin X motor domain. Confocal images of paraformaldehyde-fixed cells showed that this full-length molecule localized both to the plasma membrane and to macropinosomes, but did not localize to the tips of filopodia. This result indicates that a much larger molecule than the one used in the current study can still access and bind to membrane. Subsequently, we used the correctly sequenced full-length myosin X clone (the generous gift of Dr. R. E. Cheney, University of North Carolina) and found that in mouse myoblasts this molecule behaved exactly as reported for other cell lines (i.e. by localizing specifically to the tips of the filopodia).

Although we know that the motor domain is able to move along actin filaments in the absence of its tail, it is probable that the tail and, in particular, the PH domains are responsible for targeting myosin X to filopodia (11) and may also be important for inducing formation of new filopodia. It is hoped that the both the experimental techniques and methods of data analysis presented here will be broadly applicable to other experimental systems. In the future, the possibility of labeling different PH domains with different colored fluorescent groups (or GFP family proteins) might enable the evolution of complex signaling pathways to be observed directly inside living cells.

REFERENCES
1. Lemmon, M. A., and Ferguson, K. M. (2001) Biochem. Soc. Trans. 29, 377–384
2. Axelrod, D. (1992) in Topics in Fluorescent Spectroscopy (Lakowicz, J. R., ed) Vol. 3, pp. 289–342, Plenum Press, New York
3. Burghardt, T. P., and Axelrod, D. (1981) Biophys. J. 33, 455–467
4. Cinelli, R. A., Ferrari, A., Pellegrini, V., Tyagi, M., Giacca, M., and Belzam, F. (2000) Photochem. Photobiol. 74, 771–776
5. Garcia-Parajo, M. F., Segers-Nolten, G. M., Veerman, J. A., Greve, J., and van Hulst, N. F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7237–7242
6. Sund, S. E., and Axelrod, D. (2000) Biophys. J. 79, 1655–1669
7. Thompson, N. L., Burghardt, T. P., and Axelrod, D. (1981) Biophys. J. 33, 435–454
8. Harms, G. L., Cognet, L., Lommense, P. H. M., Blab, G. A., and Schmidt, T. (2001) Biophys. J. 80, 2396–2408
9. Mashanov, G. I., Tacon, D., Knight, A. E., Peckham, M., and Molloy, J. E. (2003) Methods 29, 142–152
10. Berg, J. S., Derfler, B. H., Pennisii, C. M., Corey, D. P., and Cheney, R. E. (2000) J. Cell Sci. 113, 3439–3451
11. Berg, J. S., and Cheney, R. E. (2002) Nat. Cell Biol. 4, 246–250
12. Yonezawa, S., Yoshizaki, N., Sano, M., Hanai, A., Masaki, S., Takizawa, T., Kageyama, T., and Moriyama, A. (2003) Dev. Growth Differ. 45, 175–185
13. Isakoff, S. J., Cardozo, T., Andreev, J., Li, Z., Ferguson, K. M., Abagyan, R., Lemmon, M. A., Aronheim, A., and Skolnik, E. Y. (1998) EMBO J. 17, 5374–5387
14. Yonezawa, S., Kimura, A., Koshiba, S., Masaki, S., Ono, T., Hanai, A., Sonta, S., Kageyama, T., Takahashi, T., and Moriyama, A. (2000) Biochem. Biophys. Res. Commun. 271, 526–533
15. Peckham, M., Miller, G., Wells, C., Zicha, D., and Dunn, G. A. (2001) J. Cell Sci. 114, 1367–1377
16. Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1991) Nature 351, 33–39
17. Simons, K., and Toomre, D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 31–39
18. Pike, L. J., and Miller, J. M. (1998) J. Biol. Chem. 273, 22298–22304
19. Caroni, P. (2001) EMBO J. 20, 4332–4336
20. van Rensen, J., and Jalili, K. (2002) Mol. Biol. Cell 13, 3257–3267
21. Varnai, P., and Balla, T. (1998) J. Cell Biol. 143, 501–510
22. Fujitawa, T., Ritchie, K., Murakoshi, H., Jacobsen, K., and Kusumi, A. (2002) J. Cell Biol. 157, 1071–1083
23. Vrijle, M., Nishimura, S. Y., Brasselet, S., Moerner, W. E., and McConnell, H. M. (2002) Biophys. J. 83, 2681–2692
24. Haugh, J. M., Codazzi, F., Tenuel, M., and Meyer, T. (2000) J. Cell Biol. 151, 1269–1280
25. Czech, M. P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 11198–11200
26. Cox, D., Berg, J. S., Cammer, M., Chinegwundoh, J. O., Dale, B. M., Cheney, R. E., and Greenberg, S. (2002) Nat. Cell Biol. 4, 469–477
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