Methods

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A Novel Biotinylated Lipid Raft Reporter for Electron Microscopic Imaging of Plasma Membrane Microdomains

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Running title:
Metabolically Biotinylated Lipid Raft Reporter

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Abbreviations: BAP, biotin acceptor peptide; EGFR, epidermal growth factor receptor; EM, electron microscopy; FRAP, fluorescence recovery after photobleaching; GFP, enhanced green fluorescent protein; HRP, horseradish peroxidase; Lck-BAP-GFP, reporter molecule containing an Lck-derived leader peptide and BAP and GFP modules; TIRF, total internal reflection fluorescence; ROI, region of interest.
ABSTRACT

The submicroscopic spatial organization of cell surface receptors and plasma membrane signaling molecules is readily characterized by electron microscopy (EM) via immunogold labeling of plasma membrane sheets. While various signaling molecules have been seen to segregate within plasma membrane microdomains, the biochemical identity of these microdomains and the factors effecting their formation are largely unknown. Lipid rafts are envisioned as submicron membrane subdomains of liquid ordered structure with differing lipid and protein constituents that define their specific varieties. To facilitate EM investigation of inner leaflet lipid rafts and the localization of membrane proteins therein, a unique genetically encoded reporter with the dually acylated raft-targeting motif of the Lck kinase was developed. This reporter, designated Lck-BAP-GFP, incorporates green fluorescent protein (GFP) and biotin acceptor peptide (BAP) modules, with the latter allowing its single-step labeling with streptavidin-gold. Lck-BAP-GFP was metabolically biotinylated in mammalian cells, distributed into low-density detergent-resistant membrane fractions, and was readily detected with avidin-based reagents. In EM images of plasma membrane sheets, the streptavidin-gold-labeled reporter was clustered in 20-50 nm microdomains presumably representative of inner leaflet lipid rafts. The utility of the reporter was demonstrated in an investigation of the potential lipid raft localization of the epidermal growth factor receptor.

Supplementary Key Words: Lck, EGFR, epidermal growth factor receptor, myristoylation, palmitoylation, metabolic biotinylation
INTRODUCTION

The lipid and protein constituents of cellular cytoplasmic membranes exhibit a high degree of structural organization on the submicron scale. Notably, a number of different cell surface receptors and associated signaling molecules are known to be localized within submicroscopic domains of the plasma membrane (1), with the concentration of some signaling molecules within “signaling microdomains” serving to enhance the efficiency of signal transduction (cf. 2), and the mutual segregation of others limiting their functional interactions (3, 4). Diverse factors are believed to underlie this microorganization of signaling molecules, including their partitioning into membrane microdomain structures (e.g. lipid rafts or caveolae), their confinement within cytoskeletal corrals, or their interactions with intra- or extra-cellular matrix (cf. 5, 6, 7). Whereas numerous biochemical, biophysical and imaging methods have been used to interrogate the submicroscopic spatial heterogeneity of membrane-associated molecules, electron microscopy (EM) has emerged as a powerful approach for its visualization. As applied in the “membrane sheet” method (3, 8, 9), immuno-EM has been elegantly exploited in the characterization of signaling microdomains involving the Ras oncoprotein (10), immune cell receptors (3), and ErbB/HER family receptors (11). Here double-immunogold labeling allows an assessment of the colocalization of distinct signaling molecules as well as their potential localization within membrane microdomains, given the availability of a suitable marker such as a resident lipid raft or caveolar protein (12, 13).

The success of immuno-EM imaging approaches is dependent upon both the identification of antibodies that efficiently and specifically label the antigens of choice and the optimization of immunolabeling conditions. To simplify the application of the immuno-EM membrane sheet method in visualizing lipid raft microdomains, we sought to identify a suitable raft marker that
(i) was localized with high specificity in lipid raft entities, (ii) was exposed on the inner surface of the cytoplasmic membrane (to facilitate labeling of inside-up membrane sheets), and (iii) could be efficiently labeled with gold-conjugated reagents. The list of lipid raft markers that have been used in various studies includes (i) membrane outer leaflet markers such as ganglioside GM1 (labeled with cholera toxin-based reagents) and various glycosylphosphatidylinositol (GPI)-anchored proteins (e.g. Thy-1 and placental alkaline phosphatase), (ii) intrinsic membrane proteins such as caveolin-1 (a marker for caveolae) and flotillins (in our experience distributed in both lipid rafts and the bulk membrane), and (iii) membrane inner leaflet markers such as the multiply acylated peripheral membrane proteins \( \Gamma_\alpha \) (dually palmitoylated) and Lck (myristoylated and dually palmitoylated). Considering our criteria for a raft marker, the extent to which various raft constituents have been characterized, and our interest in non-caveolar lipid rafts, we identified the Src family protein tyrosine kinase Lck as an ideal candidate and generated an Lck-derived reporter that could be readily labeled with avidin-biotin technology.

We present here a simplified and robust method for EM imaging of lipid rafts\(^2\) in cellular cytoplasmic membranes. This method employs a unique genetically encoded lipid raft reporter molecule that greatly facilitates the gold labeling of raft microdomains in immuno-EM membrane sheet experiments. This reporter comprises the N-terminal membrane- and lipid raft-targeting sequence of Lck, an enhanced green fluorescent protein (GFP) module for the ready assessment of reporter expression, and a metabolically biotinylated biotin acceptor peptide (BAP) module, allowing the marker to be conveniently labeled with avidin reagents such as streptavidin-gold conjugates in the context of EM imaging. We demonstrate the utility of this reporter in EM imaging of lipid rafts in plasma membrane sheets.
MATERIAL AND METHODS

Reagents, cell culture and cell transfection

Antibodies recognizing caveolin-1 (mouse monoclonal #610406, BD Transduction Labs), epidermal growth factor receptor (EGFR) (rabbit monoclonal SP9, Thermo Scientific), flotillin-2 (rabbit polyclonal H-90, Santa Cruz Biotechnology), and GFP (mouse monoclonal #N86/8, NeuroMab; rabbit polyclonal A01704, GenScript) were provided by the indicated suppliers. Transferrin receptor-specific mouse monoclonal antibody H68.4 was kindly provided by Dr. David R. Sheff. Streptavidin-horseradish peroxidase (HRP) was purchased from Perkin Elmer. Aurion BSA-C, cold water fish skin gelatin, normal goat serum, 6 nm gold particle-conjugated streptavidin, and 10 nm gold particle-conjugated F(ab')2 fragment of goat anti-rabbit IgG were obtained from Electron Microscopy Sciences. MDA-MB-231, MDA-MB-468, MCF7 and COS1 cells were purchased from the American Type Culture Collection and cultured according to their recommendations unless otherwise noted. Transient transfection of cells with plasmid cDNAs was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Design and construction of the lipid raft reporter Lck-BAP-GFP

A plasmid vector expressing the tripartite Lck-BAP-GFP reporter (Fig. 1) was constructed as follows. A cDNA encoding the 129-amino acid BAP module from the 1.3S transcarboxylase of Propionibacterium shermanii was amplified by PCR with the PinPoint Xa-1 plasmid (Promega) as template, and cloned between the BglII and SacII restriction sites of pDisplay (Invitrogen) exactly as described by Tannous et al. (14). The BAP module in this construct (kindly provided by Dr. David Motto) was subcloned via BglII and SacII restriction enzymes into pLck-GFP, an expression vector based on pEGFP-N1 (Clontech) that encodes a GFP protein with a leader peptide consisting of the 26-amino acid N-terminal membrane-targeting motif of
the human Lck kinase (15) (kindly provided by Dr. Steven H. Green). The resulting construct, pLck-BAP-GFP, encodes a reporter comprising the N-terminal Lck membrane-targeting motif, an internal BAP module, and a C-terminal GFP module. The Lck-BAP-GFP encoding sequence was directly validated by DNA sequencing.

**Western immunoblotting and streptavidin-HRP detection of biotinylated proteins following SDS-PAGE**

Proteins resolved by SDS-PAGE were detected by standard Western immunoblotting procedures or streptavidin-HRP blotting in the case of biotinylated proteins. After proteins were transferred from SDS-PAGE gels to nitrocellulose membranes, the membranes were blocked with 3% BSA in TBS. In immunoblotting, membranes were then probed with specific primary antibodies and either HRP-conjugated secondary antibodies detected by luminography with ECL Plus reagents (GE Healthcare) or IRDye secondary antibodies detected with an Odyssey imaging system (LI-COR Biosciences). Alternatively, biotinylated proteins were visualized by probing membranes with a streptavidin-HRP conjugate and ECL Plus reagents.

**Gradient fractionation of low-density detergent-resistant membranes**

Fractionation of low-density detergent-resistant membranes by sucrose density gradient flotation centrifugation was performed much as described by Rodgers et al. (16). Briefly, two ~80% confluent 150 mm dishes of MDA-MB-231 cells transiently expressing the Lck-BAP-GFP reporter were suspended in 0.75 ml of an ice-cold 1% Triton X-100 solution in TNE buffer (10 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5) supplemented with proteinase inhibitors [Protease Inhibitor Cocktail Set III (Calbiochem) and Calpain Inhibitor I (Sigma)]. This and all subsequent steps were performed at 4 C. The suspension was clarified by centrifugation at 1,300 G for 5 min, then mixed with an equal volume of 85% (w/v) sucrose in TNE buffer. The mixture
was placed in a centrifuge tube and overlaid sequentially with 6 ml of 30% (w/v) sucrose and 3.5 ml of 5% (w/v) sucrose in TNE buffer. Gradients were centrifuged at 200,000 G for 14 h. Following centrifugation, fractions were sequentially removed from the top of the gradient, and the presence of lipid raft and bulk membrane markers in the fractions was assessed by SDS-PAGE and immunoblotting with specific antibodies. Alternatively, the presence of the biotinylated Lck-BAP-GFP reporter was assessed by blotting with a streptavidin-HRP conjugate.

**Fluorescence microscopy**

For fluorescence microscopy experiments, human MCF7 or simian COS1 cells were maintained in MEM with Earle’s salts, 10% FBS, 1 x GlutaMAX-I (Gibco) or DMEM with 10% FBS (Hyclone), 1 x GlutaMAX-I, respectively, in 5% CO2 at 37 °C. Prior to experiments, cells were seeded in 35 mm dishes with glass bottoms (MatTek). The following day, cells at 40% or higher confluency were transfected with 0.3-0.5 μg of the pLck-GFP or pLck-BAP-GFP plasmid using 2-4 μL of Lipofectamine 2000 transfection reagent (Invitrogen) in OptiMEM-I medium (Gibco). After 5 h of transfection, cells were placed in normal maintenance medium and allowed to recover overnight. The next day the medium was replaced with low serum medium (1.5% FBS) and the cells examined by microscopy, either live or after fixation in 4% paraformaldehyde. Confocal images of live MCF7 cells expressing the Lck-BAP-GFP reporter were acquired with a Zeiss 510 laser scanning confocal inverted microscope with a Plan-Apochromat 63X oil immersion objective. Total internal reflection fluorescence (TIRF) images of fixed COS1 cells were acquired with a Leica AF 6000LX TIRF microscope with a 100X oil immersion objective.
Fluorescence recovery after photobleaching (FRAP) analysis of diffusional mobility

MCF7 cells grown in 35 mm culture dishes with cover glass bottoms were transfected with the pHck-GFP or pHck-BAP-GFP plasmid expression vector as described above. During FRAP measurements, cells were bathed in Hanks Buffered Salt Solution (HBSS) to maintain a steady pH. A Zeiss 510 microscope configured with a 30 °C heated stage, 63X objective, and 2X digital zoom was used for fluorescence imaging. A circular region of interest (ROI) of 5 μm diameter located in the plane of the cytoplasmic membrane was photobleached with a 488 nm argon laser (10 scans at 100% laser intensity). Using the 488 nm laser line at low power and a 505-550 nm band pass filter, pre- and post-bleach images were captured as a time series with intervals of 2 s. The Zeiss LSM Image v4.2 microscope software was used to select a control ROI, which encompassed the bleached ROI, and to obtain the integrated fluorescence intensity values for both ROIs as a function of time. To correct for the effect of gradual sample bleaching during laser scanning and instrument-related artifacts, a corrected fluorescence intensity value for the bleached ROI at each time point was calculated as the ratio of bleached ROI and control ROI intensities, which was then normalized to an initial pre-bleach intensity of 100. Using the GraphPad Prism program, the corrected data were fit by nonlinear regression with an analytical equation describing the FRAP recovery time course for the “bleached disk” experiment as derived by Soumpasis (17). The best-fit equation yielded estimates for the characteristic diffusion time ($\tau_D$), the particle diffusion constant ($D = r^2/4\tau_D$, where $r$ is the radius of the bleached disk), and the fraction of reporter molecules that are mobile (mobile fraction).

EM imaging of gold-labeled proteins in cellular plasma membrane sheets

MDA-MB-468 breast cancer cells were plated on circular glass coverslips in 35 mm culture dishes at approximately 70% confluency and cultured overnight in Leibovitz’s L-15 medium
with 10% FBS. The next day, cells were transfected with the pLck-BAP-GFP or pLck-GFP vector for 3 h in serum-free medium and incubated overnight in complete medium at 37 C. The following day, membrane sheets were isolated and fixed on EM grids by a procedure adapted from those of Wilson et al. (18) and Prior et al. (9). Briefly, cultures were washed twice with PBS, and individual coverslips were removed from the PBS, inverted, and lowered upon a group of formvar/carbon-coated EM grids that were pre-coated with poly-L-lysine. Gentle pressure was applied to the coverslip with the thumb for 10 sec. The grids were then floated off the coverslip with PBS, fixed with 2% paraformaldehyde, washed with PBS, and placed in blocking buffer [5% Aurion BSA-C (acetylated BSA), 0.1% cold water fish skin gelatin, 5% normal goat serum in PBS] for 30 min. The grids were incubated overnight with 6 nm gold-conjugated streptavidin or, in the case of double-labeling experiments, in the presence of both 6 nm gold-conjugated streptavidin and the antibody of choice (rabbit anti-EGFR or anti-GFP) diluted in incubation buffer (0.2% Aurion BSA-C in PBS). The following day, grids were washed six times with incubation buffer. In the case of double-labeling grids were further incubated with a 10 nm gold-conjugated F(ab’)2 fragment of anti-rabbit-IgG followed by six washes with incubation buffer. Grids were post-fixed with 2.5% glutaraldehyde, washed once with PBS, five times with water, stained with uranyl acetate, dried and imaged with a JEOL JEM 1230 transmission electron microscope (12,000X).

Spatial pattern analysis of particles in EM images

The spatial distribution of gold-labeled particles in EM images was analyzed by use of the Ripley’s K function statistic (cf. 9, 19). This analysis is based on Ripley’s function $K(r)$, which for a distribution of one particle type is essentially the number of particles within a distance $r$ of another divided by the average number of particles per unit area. For an ideally random particle
distribution $K(r) = \pi r^2$ for all values of $r$. The normalized statistic $\text{SQRT}[K(r)/\pi] - r$ (as plotted in Fig. 6-8) then approximates a constant value of zero for a random particle distribution, and its deviation above zero indicates a tendency for the particles in the distribution to cluster and the size scale of this clustering (20). A similar bivariate $K$ function was used to assess the tendency of particles of two different types to co-cluster. Prior to applying the $K$ function statistic, the $x$-$y$ coordinates of the centers of all gold-labeled particles in digital images were determined and 6 nm and 10 nm particles were distinguished via the application of both the automatic particle analysis and manual point selection tools in NIH ImageJ software application. Manual assessment was necessary when low contrast in images made automatic particle analysis error prone. Control experiments indicated that the 6 nm and 10 nm gold particles were readily distinguishable (see Supplemental Figures, Fig. S5). The $x$-$y$ coordinates of the particle centers were then used to evaluate $\text{SQRT}[K(r)/\pi] - r$ over a range of $r$ less than 1/5 of the image dimension via the SPPA software package generously provided by Dr. Peter Haase (19). This software uses the weighted edge correction method as originally described by Getis and Franklin (21) and modified by Haase (22). This software also allows deviations from randomness in the spatial distribution to be assessed by performing 99 simulations of random particle distributions of an equivalent density, evaluating $\text{SQRT}[K(r)/\pi] - r$ for each distribution, and graphing the envelope of the maximum and minimum deviations of the $\text{SQRT}[K(r)/\pi] - r$ values from zero.
RESULTS

A metabolically biotinylated and genetically encoded lipid raft reporter

To facilitate the gold labeling of lipid raft membrane microdomains in EM imaging studies, we developed an Lck-based lipid raft reporter construct designated Lck-BAP-GFP (Fig. 1). Like the previously characterized fluorescent reporter Lck-GFP (15), Lck-BAP-GFP incorporates the first 26 N-terminal amino acids of the human Lck protein, which includes its post-translationally modified membrane-targeting motif. Importantly, this short N-terminal sequence has been shown in the context of chimeric proteins to be myristoylated, palmitoylated and lipid-raft targeted (6, 23, 24). The Lck-BAP-GFP raft reporter developed here also incorporates BAP and GFP modules (Fig. 1). The GFP module enables the ready observation by fluorescence microscopy of the expression and subcellular localization of the genetically encoded reporter. The BAP module is known to be efficiently biotinylated by endogenous biotin ligases upon its expression in mammalian cells (14, 25), and thus confers to proteins an ability to be detected with avidin-based reagents, such as streptavidin-HRP or streptavidin-gold particle conjugates.

In our characterization of the Lck-BAP-GFP reporter, we first verified its expression and metabolic biotinylation (Fig. 2). Expression of both Lck-BAP-GFP and the parental Lck-GFP reporter in transiently transfected cultured cells was demonstrated by immunoblotting of cell lysates with a GFP antibody. Immunoblotting also showed that the migration of each protein in SDS-PAGE was consistent with its predicted molecular mass. As anticipated, Lck-BAP-GFP was readily detected upon blotting of membrane transfers of SDS-PAGE gels with a streptavidin-HRP conjugate (Fig. 2). Thus, the Lck-BAP-GFP protein appeared to be efficiently biotinylated. While the Lck-BAP-GFP reporter was the major band detected in streptavidin-HRP blots, longer-exposed blots of human cell lysates showed additional biotinylated proteins of
approximately 75 and 125 kDa (see also Fig. 5). The molecular masses of these proteins were consistent with three of the four known biotinylated proteins endogenous to mammalian cells, carboxylases of 73, 75, 125 and 270 kDa that are mitochondrial or cytosolic and thus not present in the plasma membrane (25-27). The expression of the Lck-BAP-GFP reporter in cultured cells was also examined by TIRF and confocal fluorescence microscopies (Fig. 3). TIRF microscopic images clearly showed localized expression of the GFP-tagged protein in the cellular membrane proximal to the cover glass substrate. Transverse optical sections of cultured cells obtained by confocal fluorescence microscopy showed expression of the reporter predominantly in the peripheral plasma membrane.

**Diffusional mobility of the Lck-BAP-GFP reporter**

Fluorescent reporters with multiply acylated N-termini similar to that of Lck, e.g. MyrPalm-YFP (28), have been used in biophysical investigations of the molecular diffusion of lipid raft-associated proteins. Fluorescence recovery after photobleaching (FRAP) studies have shown that, although such reporters are lipid raft-associated, they freely diffuse in the plasma membrane on the micron scale. Alternatively, fluorescence microscopic single-particle tracking experiments have shown that such reporters on the submicron scale show anomalous diffusion characterized by periods of unrestricted diffusion and episodes of restricted diffusion within submicron “transient confinement zones” (29-31). If such transient confinement zones are related to lipid rafts, these observations suggest that lipid raft microdomains are not stable structures but dynamic in nature, with constituent raft proteins free to hop between lipid raft and bulk membrane environments (29, 31) and rafts themselves possibly representing a transient coalescence of a liquid ordered membrane phase (32).
The presence of the GFP module in the Lck-BAP-GFP reporter makes it applicable in such biophysical approaches. As an example, to determine whether the Lck-BAP-GFP reporter behaved as previously characterized Lck-like reporters, we examined the macroscopic diffusion of Lck-GFP and Lck-BAP-GFP in the cellular membrane by FRAP (Fig. 4). Both Lck-GFP and Lck-BAP-GFP showed unrestricted diffusion on the micron scale with apparent diffusion constants of 0.44 and 0.32 μm²/sec, respectively, and mobile fractions close to 1.0. These diffusional characteristics closely agree with the results of previous FRAP studies of reporters with Lck-like membrane anchors (28, 33). The high mobile fractions observed suggest that the diffusion of both reporters is unrestricted on the time-scale of these measurements, and importantly, that the incorporation of the BAP module into Lck-BAP-GFP does not cause the interaction of Lck-BAP-GFP with plasma membrane or cytoskeletal matrix proteins in a manner that significantly impedes its diffusion.

**Distribution of Lck-BAP-GFP into low-density detergent-resistant membrane fractions**

Appending the N-terminal membrane-targeting motif of Lck to a reporter molecule of interest can confer a lipid raft localization to that reporter. In biochemical fractionation approaches, various Lck-based reporters have been found to distribute into detergent-resistant membrane fractions of low-density. Although the relationship of such biochemically defined detergent-resistant membranes to lipid raft microdomains existing in intact cell membranes is unclear (34), detergent-resistant membranes are enriched in various lipid raft constituents (cf. 35, 36). We sought to verify that the metabolically biotinylated Lck-BAP-GFP reporter would like other characterized Lck-based probes and the Lck protein itself distribute into detergent-resistant membranes (16, 23). Figure 5 shows a density gradient fractionation of membrane proteins from MDA-MB-231 breast cancer cells expressing the Lck-BAP-GFP reporter following their
treatment with cold Triton X-100. In such gradient flotation experiments, detergent-resistant membranes and lipid raft-associated proteins are expected to migrate to fractions of low buoyant density, while cytoplasmic proteins and detergent-solubilized non-raft membrane proteins will remain in the densest gradient fractions. Consistent with this expectation, immunoblotting showed the concentration of the lipid raft proteins caveolin-1 and flotillin-2 in low-density gradient fractions (fractions 5 and 6), whereas the non-raft membrane protein transferrin receptor was found exclusively in the bottom gradient fractions (fractions 14-16). While some of the biotinylated Lck-BAP-GFP reporter was found in the cytosolic/solubilized membrane fractions, it was present at highest concentrations in the low-density detergent-resistant membrane fractions. Because the association of Lck and Lck-derived reporters with detergent-resistant membranes is known to be dependent upon both myristoylation and palmitoylation (16, 23), these results indicate that the Lck-BAP-GFP reporter is subject to both translational modifications. We note that in the cytosolic/solubilized membrane fractions (fractions 14-16), the Lck-BAP-GFP protein migrated as a doublet with a higher mobility species evident therein. This second species might represent an Lck-BAP-GFP protein with incomplete post-translational modification, and thus devoid of a fully functional membrane-targeting motif. Also detected in the cytosolic/solubilized membrane fractions were biotinylated proteins of approximately 75 and 125 kDa, which likely represent endogenously biotinylated mitochondrial carboxylases (see above).

In agreement with earlier reports that the EGFR is solubilized in cold Triton X-100 (36, 37), this putative lipid raft resident protein was also found in the solubilized gradient fractions. This suggested that if the EGFR is indeed a lipid raft resident, it likely resides in rafts of different
biochemical composition than of those into which the Lck-BAP-GFP reporter partitions (see Discussion).

EM imaging of the gold-labeled Lck-BAP-GFP reporter in plasma membrane sheets

Given the ease of its detection with avidin-based technologies, the Lck-BAP-GFP reporter described herein should have diverse applications in the investigation of lipid raft entities, for example its use as a marker of lipid rafts in biochemical fraction experiments (Fig. 5). We envisioned it would be especially useful in EM characterization of protein micro-localization in plasma membrane sheets (3, 8, 9), wherein proteins of interest would be labeled with immunogold reagents and the biotinylated Lck-BAP-GFP lipid raft reporter with a streptavidin-gold particle conjugate. Thus, we next sought to demonstrate that the Lck-BAP-GFP reporter in cellular membranes could be effectively labeled with streptavidin-gold and its spatial distribution imaged by EM. To this end, membrane sheets derived from cultured cells ectopically expressing the Lck-BAP-GFP reporter were immobilized on polylysine-coated EM grids, and the membranes were fixed and then labeled in a single step with a 6 nm gold particle-conjugated streptavidin reagent (Fig. 6). A representative image of gold-labeled Lck-BAP-GFP reporters in MDA-MB-468 breast cancer cell membranes is shown in Fig. 6A. A fairly dense labeling of the plasma membrane surface was generally seen, with an apparent clustering of the gold labels in microdomains of less than ~100 nm. The companion Fig. 6B is a control image representative of membranes from cells expressing a non-biotinylated raft reporter Lck-GFP and subjected to the same streptavidin-labeling procedure. In this case and as quantified in Fig. 6C, a dramatically reduced labeling was evident, which indicated that the Lck-BAP-GFP reporter can be labeled in plasma membrane sheets with good specificity. This was anticipated given the high specificity
of avidin-labeling technologies and that biotinylated proteins endogenous to mammalian cells are mitochondrial.

The spatial distribution of Lck-BAP-GFP reporters on the membrane surface and their apparent tendency to cluster were analyzed by use of the Ripley’s $K$ function statistic (Fig. 6D). By this analysis of the center-to-center inter-particle distances, the distribution of gold-labeled Lck-BAP-GFP reporters in EM images was seen to be nonrandom, with a clustering of the reporters prominent on the size scale of 20-50 nm. This is similar to the size of Ras microdomains identified by Prior et al. in cellular plasma membranes (10), and consistent with the proposed size of lipid raft structures (35).

To further validate the streptavidin-gold labeling strategy, we performed double-labeling experiments in which membrane sheets from Lck-BAP-GFP-expressing cells were labeled with both streptavidin-gold and a GFP-specific antibody detected with a conventional immunogold reagent. The patterns of labeling by streptavidin-gold and the immunogold conjugate independently showed clustering on the scale of 20-50 nm (Fig. 7). Importantly, there was significant co-clustering of the two labels as shown by a bivariate Ripley’s $K$ function analysis (Fig. 7D). Depending upon the efficiencies of labeling of the Lck-BAP-GFP reporter with each of the two detection reagents and the number of reporter molecules present in individual rafts, either a perfect or partial overlap of streptavidin-gold and immunogold-labeled clusters would be predicted. Thus, we interpret the co-clustering of streptavidin-gold and anti-GFP/immunogold labeling (Fig. 7D) as evidence that both detection reagents effectively label the Lck-BAP-GFP reporter.

We note that the 6 nm gold-conjugated streptavidin reagent displayed some tendency to self-cluster in control experiments in which the reagent was directly deposited on an EM grid
(see Supplemental Figures, Fig. S1 and S2). The observed clustering of Lck-BAP-GFP was much more extensive and was also observed by conventional immunogold labeling with an alternative gold-conjugated reagent that showed no tendency to self-cluster (see Fig. 7 and Fig. S3 and S4 in Supplemental Figures)

**Application of the Lck-BAP-GFP reporter as a lipid raft marker in EM imaging**

The localization of the expressed Lck-BAP-GFP reporter in low-density detergent-resistant membrane fractions and its observed clustering in plasma membrane domains of 20-50 nm are consistent with the reporter being targeted to lipid rafts, i.e. membrane microdomains with lipid composition distinct from the bulk membrane and with a liquid ordered structure (see Discussion). Given its ease of detection with streptavidin reagents, we anticipate that this reporter will be a useful inner leaflet lipid raft marker for both biochemical experiments and various imaging applications. To demonstrate the utility of Lck-BAP-GFP as a raft marker in EM imaging, we applied it in assessing the potential lipid raft localization of epidermal growth factor receptor (EGFR) molecules in breast cancer cell plasma membranes. Here, membrane sheets derived from cultured MDA-MB-468 breast cancer cells were simultaneously subjected to conventional immunogold labeling of the EGFR (with 10 nm gold particles) and streptavidin-gold labeling of the ectopically expressed Lck-BAP-GFP reporter (with 6 nm gold particles). As shown in the representative image in Fig. 8A, both the EGFR and Lck-BAP-GFP were effectively gold-labeled and the two labeled species readily distinguished.

When the spatial distributions of the labeled Lck-BAP-GFP and EGFR molecules were independently analyzed (Fig. 8B and C), each showed significant deviation from randomness and clustering on the submicron scale. While the Lck-BAP-GFP reporter again showed clustering most predominant in the size range of 20-50 nm (compare Fig. 6D and 7B), the EGFR showed
clustering over a broader size range (25-150 nm). Such submicron-sized clustering of the EGFR in cellular plasma membranes has been previously characterized by both immuno-EM (11, 38) and single molecule-resolution fluorescence microscopy (39), although the factors responsible for the establishment of these microdomains are the subject of continuing investigation (7, 38-40). By analyzing the co-clustering of the EGFR and Lck-BAP-GFP reporter with the bivariate Ripley’s $K$ function statistic (Fig. 8D), we addressed the issue of whether these observed EGFR receptor microdomains represent lipid raft entities. In this and four repeated experiments (data not shown), there was no significant co-clustering of the EGFR and the lipid raft marker beyond what might be explained by a random coincidence of localization. We conclude that EGFR microdomains in MDA-MB-468 breast cancer cell membranes do not represent lipid raft species, at least not of the type into which a multiply acylated inner leaflet raft protein such as Lck would partition.
DISCUSSION

Lck is among those Src family protein tyrosine kinases having an N-terminal membrane-targeting motif subject to both myristoylation and palmitoylation. Thus, the N-terminal sequence of the nascent Lck peptide (MGCGCS in human Lck) is post-translationally modified by proteolytic removal of the initiating methionine, followed by myristoylation of Gly-2 and palmitoylation of at least one of the two cysteine residues Cys-3 and Cys-5 (16, 41). Post-translational modification with multiple saturated fatty acid motifs confers to some proteins an affinity for lipid raft domains, as demonstrated by a number of biochemical, biophysical and imaging approaches (cf. 42). In the case of Lck, a variety of studies indicate that its N-terminal targeting motif is sufficient to effect its localization in lipid rafts (6, 16, 23, 24, 41). Indeed, biophysical studies using fluorescent reporters with Lck-like targeting motifs have provided crucial evidence for the existence of lipid rafts in intact cells (6, 24, 43).

For some time the subject of controversy, lipid rafts are generally envisaged as small (<200 nm) membrane microdomains enriched with sphingolipids and cholesterol and possessing a more ordered structure than the bulk plasma membrane (which apparently underlies the enhanced affinity of proteins with saturated fatty acid modifications for lipid rafts). The characterization of lipid raft entities has been confounded by the limited resolution of classical light microscopy, their intractability to biochemical fractionation, and the potentially transient nature of their formation (cf. 34, 44). There is also significant diversity among lipid raft species, with differing raft markers having been shown to occupy distinct domains (4, 45). Thus, while EM examination of the spatial distributions of the plasma membrane outer leaflet lipid raft constituents sphingomyelin and ganglioside GM1 showed them each clustering within submicron domains, the observed sphingomyelin- and ganglioside GM1-rich microdomains were
nonidentical (45). The paucity of well characterized lipid raft reporters, the recognized diversity of lipid raft species, and the significant interest in characterizing the spatial organization of plasma membrane-associated signaling molecules (many of which are peripheral membrane proteins or transiently associated with the plasma membrane inner surface) highlight the need for well characterized probes of membrane inner leaflet microdomains. The Lck-based lipid raft reporter described herein is thus expected to be particularly useful in view of its targeting to inner leaflet membrane microdomains (domains that are possibly distinct from those interrogated with commonly used outer leaflet reporters such as GPI-anchored proteins or ganglioside GM1-binding cholera toxin) and also because of its amenability to the immuno-EM membrane sheet imaging methodology. The ease of its detection with avidin-based technology should make the Lck-BAP-GFP reporter also useful in the context of biochemical approaches for the study of lipid rafts.

The present work demonstrates the particular utility of the genetically encoded Lck-BAP-GFP reporter in the characterization of lipid raft entities by EM. The reporter was readily expressed in cultured cells and metabolically biotinylated therein, as indicated by its detection with streptavidin-HRP in blots of SDS-PAGE gels (Fig. 2) and streptavidin-gold conjugates in EM imaging (Fig. 6-8). In membrane fractionation experiments, the reporter as expected was found in low-density detergent-resistant membrane fractions along with traditional lipid raft markers such as caveolin-1 and flotillin-2 (Fig. 5). When the spatial distribution of the Lck-BAP-GFP reporter in the plasma membrane was examined by EM imaging of streptavidin-gold-labeled membrane sheets, the reporter showed a non-random localization with obvious clustering on the scale of 20-50 nm (Fig. 6-8). The submicron-scale clustering of the Lck-BAP-GFP reporter, along with its observed fractionation in low-density detergent-resistant membranes, is
consistent with its anticipated lipid raft localization. Nonetheless, in agreement with earlier studies of raft-associated proteins (cf. 33), the Lck-BAP-GFP reporter showed free diffusion on the micron scale (Fig. 4), suggesting that its association with lipid rafts is transient in nature or that lipid rafts themselves are dynamic in structure. Interestingly, related single-particle tracking studies of Lck-based fluorescent reporters have shown their diffusion on the scale of one micron, with periodic restrictions of diffusion within submicron zones of confinement possibly representative of lipid raft entities (29, 30, 46).

The primary goal of this study was to create a genetically encoded lipid raft marker that would facilitate EM investigations of membrane protein micro-localization and lipid raft association. We made a first application of this reporter in an EM investigation of the proposed lipid raft localization of the EGFR (Fig. 8). While a number of biochemical fractionation studies have shown that the EGFR resides to a significant extent in low-density membrane fractions believed to represent lipid rafts (36, 37, 47-50), our results indicated that the microdomains of EGFR localization observed by immuno-EM imaging of plasma membrane sheets (11, 38) are not identical to those in which the inner leaflet raft marker Lck-BAP-GFP resides. We note that two previous EM studies, which used cholera toxin B-HRP staining of ganglioside-GM1 (40) or immunogold labeling of placental alkaline phosphatase (50) in ultrathin cryosections, showed a limited colocalization of EGFR molecules with plasma membrane regions containing outer leaflet raft markers. Also, multiple studies employing both biochemical techniques and EM imaging have shown that the EGFR, while present in low-density membrane fractions also rich in caveolin-1, does not reside to a significant extent within caveolae (37, 49, 50). The present study suggests that EGFR microdomains observed by immuno-EM are also not identical with lipid rafts that are labeled with an Lck-based reporter.
Our observation that the EGFR was apparently solubilized in cold Triton X-100 and was not present in the Triton X-100-resistant, caveolin-1- and Lck-BAP-GFP-enriched lipid raft fractions (see Fig. 5) is consistent with earlier investigations of the detergent-dependent solubility the EGFR-containing lipid raft species (36, 37). In particular, Pike et al. (36) found that lipid rafts resistant to cold Triton X-100 (in which the Lck-BAP-GFP reporter was here found to be enriched) have a lipid composition distinct from that of EGFR-containing lipid rafts isolated using alternative detergents or a detergent-free method. This bolsters our conclusion that EGFR-containing membrane microdomains are distinct from those into which the Lck-BAP-GFP reporters partitions. Nonetheless, the biochemical characteristics of EGFR microdomains and the factors responsible for their formation remain open questions.

The novel Lck-BAP-GFP reporter described herein should be a valuable tool for the investigation of plasma membrane lipid rafts. This reporter is readily detected in both biochemical fractionation and EM imaging applications by virtue of its constitutive biotinylation when expressed in mammalian cells. Our observation of Lck-BAP-GFP clustering within submicron domains suggests it might indeed reside in cholesterol-rich liquid ordered microdomains as have been observed in model membrane systems. However, we reiterate that the lipid raft species in which this inner leaflet marker resides are likely distinct from those labeled by other raft reporters. Thus, the Lck-BAP-GFP reporter should not be considered a marker of lipid rafts in general, but a specific marker of rafts for which a multiply acylated inner leaflet protein would have affinity. We envision that additional lipid raft reporters exploiting the same avidin-based detection technology but derived from distinct raft resident proteins (e.g. caveolin or flotillin proteins) would be of much utility in characterizing the diversity of lipid raft species.
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EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine

FOOTNOTES

2Given the diversity of lipid raft species that has been indicated by studies of differing lipid raft markers (cf. 4, 45), we intend that the term “lipid raft,” when used in the context of the Lck-BAP-GFP reporter and imaging method described herein, be understood to mean those membrane microdomain entities into which the multiply acylated reporter partitions. These microdomains are likely to be related to cholesterol-rich lipid rafts as have been defined biochemically.
FIGURE LEGENDS

Fig. 1. Schematic structure of a metabolically biotinylated lipid raft reporter, Lck-BAP-GFP.

For the purpose of imaging lipid raft microdomains in cellular plasma membranes, a genetically encoded lipid raft-targeted reporter construct was developed that is metabolically biotinylated and thus detectible with avidin reagents. Schematic structure of the Lck-BAP-GFP reporter shows: Lck-N, the N-terminal lipid raft-targeting sequence of the Lck kinase with sites of myristoylation and dual palmitoylation indicated; BAP, the biotin acceptor peptide sequence that is metabolically biotinylated by endogenous mammalian biotin ligases; GFP, enhanced green fluorescent protein module for detection by fluorescence microscopy.

Fig. 2. Expression and metabolic biotinylation of the Lck-BAP-GFP reporter. MDA-MB-468 breast cancer cells were lysed 24 h following transfection with either the pLck-GFP or pLck-BAP-GFP vector, and cell lysate samples (15 μg protein) subjected to SDS-PAGE and immunoblotting with anti-GFP (left). Both the 32 kDa Lck-GFP and 45 kDa Lck-BAP-GFP reporters were detected. Specific biotinylation of Lck-BAP-GFP was shown by blotting with a streptavidin-HRP conjugate and enhanced chemiluminescence detection (right).

Fig. 3. Fluorescence imaging of the genetically encoded Lck-BAP-GFP reporter expressed in cultured cells. A: Cultured COS1 cells expressing Lck-BAP-GFP were fixed 48 h after transfection, and the GFP signal imaged by total internal reflection fluorescence (TIRF) microscopy. Shown is a representative image in which significant expression of the reporter proximal to the cell surface is evident. B: MCF7 breast cancer cells expressing Lck-BAP-GFP were imaged by laser scanning confocal microscopy. A 0.8 μm transverse optical section of a representative Lck-BAP-GFP-expressing cell shows GFP fluorescence localized primarily in the peripheral cellular membrane. Scale bars: 25 μm.
Fig. 4. FRAP analysis of the diffusional mobility of Lck-derived reporters in the cellular plasma membrane. Cultured MCF7 cells were transfected with an Lck-GFP or Lck-BAP-GFP expression vector as indicated and the diffusional mobility of the reporters assessed by FRAP (see Materials and Methods). A: Representative image showing the plasma membrane fluorescence of the Lck-BAP-GFP reporter immediately after photobleaching of a 5 μm circular area. Superimposed are the bleached disk (white circle) and control (surrounding white tracing) ROIs used in the FRAP analysis. B: Quantitative analysis of the FRAP recovery for the experiment depicted in A. Integrated fluorescence intensities within the bleached and control ROIs and the corrected bleached ROI intensity data are shown. Curve fitting of the corrected data yielded the best-fit theoretical recovery curve depicted and estimates for the diffusion constant, $D$, and mobile fraction. C-D: Values of $D$ (C) and the mobile fraction (D) from individual FRAP experiments are graphed in aggregate, with the mean values and standard errors of the mean indicated by bars.

Fig. 5. Identification of Lck-BAP-GFP in low-density detergent-insoluble lipid raft fractions of cellular membranes. In a conventional lipid raft fractionation protocol, MDA-MB-231 cells expressing the Lck-BAP-GFP reporter were suspended in ice-cold 1% Triton X-100 and the suspension subjected to density gradient flotation centrifugation (see Materials and Methods). Gradient fractions were numbered in order of increasing density, and the presence of membrane markers in the fractions assessed by SDS-PAGE and immunoblotting with specific antibodies. The biotinylated Lck-BAP-GFP reporter was detected by blotting with a streptavidin-HRP conjugate (SA-HRP). The traditional lipid raft markers caveolin-1 (Cav-1, 22 kDa) and flotillin-2 (Flot-2, 47 kDa) were identified in low-density detergent-insoluble fractions, whereas the EGFR (180 kDa) and the bulk membrane (non-raft) marker transferrin receptor (TfR, 85 kDa)
were found in the high-density solubilized protein fractions. Streptavidin-HRP blotting showed
the presence of the biotinylated Lck-BAP-GFP marker (45 kDa) in both lipid raft and solubilized
fractions and the presence of previously identified endogenously biotinylated mitochondrial
proteins of 75 and 125 kDa in the solubilized fractions.

**Fig. 6.** EM imaging of the streptavidin-gold-labeled Lck-BAP-GFP reporter in plasma
membrane sheets. Cellular plasma membrane sheets from MDA-MB-468 breast cancer cells
expressing either the Lck-BAP-GFP or Lck-GFP reporter were isolated and prepared as for
standard immunogold labeling, but labeled with a 6 nm streptavidin-gold conjugate. Labeled
membranes were imaged by transmission EM at 12,000X (see *Materials and Methods*). A: EM
image of a streptavidin-gold-labeled plasma membrane sheet from an Lck-BAP-GFP-expressing
cell. Submicron-sized clusters of the gold-labeled reporter were evident (see arrow). B: EM
image of streptavidin-gold-labeled membrane of a control cell expressing the Lck-GFP reporter.
C: Quantification of labeling of Lck-BAP-GFP-expressing versus Lck-GFP-expressing (control)
cells in repeated experiments. Shown are the mean and standard error of the mean for 11
experiments in which particles in equivalent membrane areas were counted. D: Representative
Ripley’s *K* function analysis of the clustering of gold particles in EM images of labeled Lck-
BAP-GFP-expressing MDA-MB-468 cell membranes. The image analyzed (a cropped version
of which is shown in A) corresponded to a total membrane area of 1443 nm x 1443 nm. The
graph displays the evaluated function \( \text{SQRT}[K(r)/\pi] – r \) for the particle distribution (–––). Also
shown are the envelopes of the maximum and minimum deviations of the evaluated functions
(····) for 99 randomly generated distributions of the same number of particles. A non-random
distribution was evident with clustering on the dimension of 20-50 nm.
Fig. 7. Double-labeling of the Lck-BAP-GFP reporter with streptavidin-gold and conventional immunogold reagents. Membrane sheets from MDA-MB-468 cells expressing Lck-BAP-GFP were labeled with both a GFP-specific rabbit polyclonal antibody/10 nm gold-conjugated secondary antibody pair and a 6 nm gold-conjugated streptavidin reagent. A: Representative EM image (cropped version of the larger analyzed image) of 6 nm streptavidin-gold (arrow) and anti-GFP/10 nm gold (arrowhead) labeling in a plasma membrane sheet (12,000X). B-D: Ripley’s $K$ function plots (–––) characterizing the spatial distributions of streptavidin-gold (B) and anti-GFP/immunogold (C) labeling, and bivariate $K$ function plot (–––) characterizing the co-clustering of streptavidin-gold and anti-GFP/immunogold (D). The envelopes of the evaluated $K$ functions for 99 randomly generated distributions of the same numbers of particles are also indicated (⋯⋯). Both streptavidin-gold and anti-GFP/immunogold individually showed clustering on the scale of 20-50 nm, and the bivariate analysis showed a co-clustering of the two labels on a similar size scale.

Fig. 8. Application of the Lck-BAP-GFP reporter in assessing the potential lipid raft localization of the EGFR. Membrane sheets from MDA-MB-468 cells expressing a high level of endogenous EGFR and the ectopic Lck-BAP-GFP lipid raft marker were analyzed by EM after gold labeling of the EGFR and Lck-BAP-GFP proteins. The EGFR was immunolabeled with a rabbit monoclonal antibody and 10 nm gold-conjugated secondary antibody, and the biotinylated raft marker was labeled with a 6 nm gold-conjugated streptavidin reagent. A: Representative transmission EM image (cropped version of the larger analyzed image) of 10 nm gold-labeled EGFR (arrowhead) and 6 nm gold-labeled Lck-BAP-GFP (arrow) proteins in a plasma membrane sheet (12,000X). B-C: Ripley’s $K$ function analysis of the clustering of the labeled Lck-BAP-GFP reporter (B) and EGFR (C). D: The evaluated bivariate $K$ function (–––) for the
Lck-BAP-GFP and EGFR particle distributions in the representative image fell largely within the envelope of 99 simulated random distributions (⋯⋯), which indicated little tendency for the EGFR and raft reporter to co-cluster.
Figure 2
Figure 4
Figure 5

[Image of a gel electrophoresis blot with markers for protein bands at 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 15 kDa, 50 kDa, 37 kDa, 25 kDa, 100 kDa, 75 kDa, and 250 kDa. Proteins labeled: SA-HRP, Cav-1, Flot-2, Tfr, EGFR.]
Figure 6
Figure 7
Figure 8

(A) 10 nm Particle Distribution (EGFR)

(B) 6 nm Particle Distribution (Lck-BAP-GFP)

(C) 6 nm and 10 nm Particle Co-clustering