# Reversible penetration of α-glutathione S-transferase into biological membranes revealed by photosensitized labelling *in situ*

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Fluorescent lipid analogue 3,3′-dioctadecyloxacarbocyanine incorporated into biological membranes was used to induce photoactivation of a hydrophobic probe 5-[$^{125}$I]iodonaphthyl-1-azide ($^{125}$INA) by energy transfer and to thereby confine subsequent radiolabelling of proteins to the lipid bilayer. This approach was applied in bovine chromaffin cells to discover cytosolic proteins that reversibly penetrate into membrane domains. α-Glutathione S-transferase (α-GST) was identified as the only labelled protein in bovine chromaffin-cell cytosol, indicating that it inserts reversibly into the membrane lipid bilayer. The selectivity of the labelling towards the lipid bilayer is demonstrated by showing that influenza virus haemagglutinin becomes labelled by $^{125}$INA only after the insertion of this protein into the target membrane. The molar $^{125}$INA:protein ratio was used as a quantitative criterion for evaluation of the penetration of proteins into the membrane lipid bilayer. This ratio was calculated for four integral membrane proteins and four soluble proteins that interact with biological membranes. The values for four integral membrane proteins (erythrocyte anion transporter, multidrug transporter gp-170, dopamine transporter and fusion-competent influenza virus haemagglutinin) were 1, 8, 2 and 2, respectively, whereas for soluble proteins (annexin VII, protein kinase C, BSA and influenza virus haemagglutinin) the values were 0.002, 0, 0.002 and 0.02, respectively. The molar ratio for α-GST was found to be 1, compatible with the values obtained for integral membrane proteins.

## INTRODUCTION

Studying dynamic interactions between proteins and biological membranes requires methodologies capable of high time and space resolution. Several biophysical and biochemical techniques have been used in these studies [1–8]. These methods are efficient at monitoring fast interactions between proteins and membranes, but they are indirect, often require the use of isolated components and cannot be used for the identification of unknown proteins. This constitutes a significant obstacle in studying protein–membrane interactions *in situ* and in establishing the localization of a protein with respect to the membrane lipid bilayer. Hydrophobic labelling has been used to identify segments of integral membrane proteins that make direct contact with the lipid bilayer [9]. The photoreactive compound 5-[$^{125}$I]iodonaphthyl-1-azide ($^{125}$INA) is a hydrophobic membrane probe with high retention in lipid bilayer. When photoactivated, it selectively tags protein domains embedded in the lipid phase of native biological membranes [10,11]. The tendency of $^{125}$INA to diffuse from the membrane to hydrophobic sites located on soluble proteins compromises its efficiency, especially in applications that need to establish the orientation of proteins that undergo transition from soluble to membrane-bound form. These problems are eliminated by confining the photoactivation of $^{125}$INA to specific membrane compartments by site-directed photosensitization [12]. In this approach, a variety of chromophores are used as photosensitizers which, upon irradiation with visible light, induce photoactivation of $^{125}$INA through energy transfer. The mechanisms of energy transfer require close proximity between a donor chromophore and an acceptor probe. Therefore, subsequent to the activation, radiolabelling of proteins and lipids is also confined to the vicinity of the chromophore [12]. Site-directed photosensitized labelling has been successfully applied *in situ* to answer a variety of biological questions [12–15]. In this study, we use the fluorescent lipid analogue 3,3′-dioctadecyloxacarbocyanine (DiO) as a photosensitizer to target labelling of proteins exclusively to the lipid bilayer of biological membranes. We implement this approach for identification *in situ* of soluble proteins in the cell cytosol that can penetrate reversibly into the lipid bilayer. We find that in bovine chromaffin cells, α-glutathione S-transferase (α-GST) reversibly inserts protein domains into the hydrophobic phase of cell membranes. GSTs are a family of enzymes involved in a variety of detoxification reactions. They catalyse the conjugation of glutathione to a number of hydrophobic electrophilic substrates, catalyse glutathione-dependent isomerizations, have peroxidase activity and act as binding proteins for a range of non-substrate ligands [16,17]. Some endogenous compounds, such as phospholipid peroxides [18], $\Delta^5$-3-ketosteroids [19] and prostaglandin $H_2$ [20], have been shown to be substrates for GSTs in solution. Steroids, bilirubin and bile acids can bind non-covalently to GSTs [21]. Nevertheless, the mechanism of binding of hydrophobic substrates to GSTs *in situ* is not entirely understood.

## EXPERIMENTAL

### Materials

X31 (A/Hong Kong/1/68) strain of influenza virus was generously donated by Dr. Robert Blumenthal (NCI, Bethesda,

Abbreviations used: $^{125}$INA, 5-[$^{125}$I]iodonaphthyl-1-azide; DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; GST, glutathione S-transferase; PKC, protein kinase C; HA, haemaggluttinin.

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U.S.A.). Polyclonal goat antiserum to X31 was generously donated by Dr. Ari Helenius (Yale University School of Medicine, Yale, U.S.A.). DiO was from Molecular Probes (Eugene, OR, U.S.A.). $^{125}$INA (1 mCi/$\mu$mol) was from Lofstrand Laboratories (Gaithersburg, MD, U.S.A.). Protein kinase C (PKC) was from Calbiochem (La Jolla, CA, U.S.A.), BSA (fraction V, radioimmunoassay grade) was from Sigma (St. Louis, MO, U.S.A.) and fatty acid ultra-free BSA (fraction V) was from Boehringer-Mannheim (Indianapolis, IN, U.S.A.).

### Preparation of bovine adrenal chromaffin cells and isolated chromaffin granules

Bovine adrenal chromaffin cells were dissociated from fresh bovine adrenal glands by collagenase digestion as described previously [22]. Bovine chromaffin cells were maintained in suspension culture in bacterial Petri dishes at a density of $2 \times 10^4$ cells/dish and used within 1–3 days. Isolated chromaffin granules were prepared from disrupted bovine adrenal glands as described elsewhere [22].

### Loading of chromaffin cells and chromaffin granules with DiO and $^{125}$INA

Cultured chromaffin cells were washed twice with Krebs medium and incubated with DiO (30–45 $\mu$g/ml final concentration) in buffer C (Sigma) according to the manufacturer's instructions. DiO-containing cells were washed twice with serum-containing medium to remove unbound DiO and then washed with Krebs medium. $^{125}$INA (1–5 $\mu$Ci) was added to the DiO-containing cells and incubated for 5 min at room temperature under dim light. The cells were then washed twice with Krebs medium. Reduced glutathione (20 mM), a scavenger of long-lived excited-state photoproducts of $^{125}$INA, was present at all stages of the experiment to prevent diffusion of these species from their site of formation into the other proteins. Isolated bovine chromaffin granules were treated as intact chromaffin cells except that PBS was used instead of Krebs medium.

### Photolabelling of chromaffin cells and isolated chromaffin granules with $^{125}$INA

Direct UV activation of $^{125}$INA was performed using a 200 W HBO Mercury lamp. The samples were placed in 5 mm × 60 mm glass tubes 5 cm from the source. Irradiation was performed through two 0–52 filters (Kopp Glass, Inc., Pittsburgh, PA, U.S.A.) which allow transmittance ($\lambda$) > 350 nm using a water filter for heat absorption. Irradiation of the samples was carried out for 90 s. Photosensitized activation of $^{125}$INA was done using a monochromatic illumination system consisting of a 1000 W xenon lamp as a light source. The lamp was coupled to a GM-252 grating monochromator (Spectral Energy Corp., Ramsey, U.S.A.) and monochromatic light was collimated into a sample chamber in which individual 6 mm × 50 mm sample tubes were placed. The monochromator was set to allow transmission of monochromatic light at 480 nm with a band width of 10 nm and a slit height of 10 mm. Illumination was carried out for 10 min.

### Separation of cytosolic and membrane fractions of chromaffin cells

The chromaffin cells, either non-treated or loaded with $^{125}$INA + DiO and irradiated, were washed twice with Krebs medium, lysed in hypotonic medium (10 mM Tris/HCl, pH 7.5, 1 mM EGTA/0.2 mM $MgCl_2$/100 $\mu$g/ml leupeptin/100 $\mu$M pepstatin/10 $\mu$g/ml DNAse), and put through three cycles of freezing and thawing (solid $CO_2$ −37 °C water bath), 10 min each cycle. The cytosolic and membrane fractions were separated by centrifugation at 10000 ***g*** for 30 min at 4 °C. The supernatant was collected and subjected to ultracentrifugation in an Airfuge (Beckman) at 148000 ***g*** for 1 h at 4 °C.

### Quantitative analysis of the labelled proteins

Three identical samples of labelled proteins obtained in the same experiment were loaded on 10–12 % SDS/PAGE (Laemmli), separated and stained with Coomassie Brilliant Blue stain. The gels were dried and exposed to PhosphorImager screens for 24 h. The bands corresponding to the proteins of interest were excised from the gels and counted in a gamma counter. The standard deviation for the values obtained did not exceed 2 %.

### Purification of the $^{125}$INA-labelled 28 kDa protein from bovine chromaffin cell cytosol

The cytosol of photosensitized chromaffin cells was dialysed against 20 mM Tris/HCl, pH 7.4, overnight. The cytosol was subjected to FPLC using an anion-exchange column, MonoQ (Pharmacia, Uppsala, Sweden), and the proteins were eluted from the column with 0–0.5 M ammonium acetate gradient. The 28 kDa protein was detected in flow-through eluent by following the radioactivity. Flow-through eluent was passed once more on the MonoQ column. The flow-through fractions containing radioactivity were pooled together, concentrated on Centriprep-10 (Filtron, Northborough, U.S.A.), and applied to a gel-filtration ACA 54 (LKB, Bromma, Sweden) column. Radioactive fractions were collected and dialysed against 20 mM sodium phosphate buffer, pH 7.2. The sample was subsequently subjected to HPLC using a reverse-phase $C_{18}$ column (D pak; Waters, Milford, U.S.A.), and the proteins were eluted with a linear gradient of 0–100 % acetonitrile. The 28 kDa protein was eluted in 50 % acetonitrile. This fraction was lyophilized, resuspended in water and used for further analysis.

### Identification of 28 kDa protein as $\alpha$-GST

The 28 kDa protein, purified to homogeneity as described earlier, was subjected to SDS/PAGE, stained with Coomassie Brilliant Blue, and excised from the gel. The protein was digested with trypsin, generated peptides were isolated by HPLC and two peptides were microsequenced at W. M. Keck Foundation Biotechnology Resource Laboratory (New Haven, CT, U.S.A.). A homology search for the peptide sequences obtained identified the 28 kDa protein as $\alpha$-GST.

### Quantitative analysis of the labelling of $\alpha$-GST

Purified $\alpha$-GST was subjected to SDS/PAGE, and quantitative analysis was performed as described earlier. The amount of GST in the band was determined by densitometry using a BSA calibration curve. The image was taken by a COHU 6515 electronic 35 mm camera and analysed using NIH Image 1.57 software.

### Incubation of influenza virus with chromaffin cells

The X31 strain of influenza virus was bound to chromaffin cells preloaded with DiO and $^{125}$INA in PBS for 20 min at 4 °C. Citric acid (0.5 M) was added to the samples to reduce the pH to 5.0. After 15 min of incubation at 37 °C, the pH was neutralized to 7.4 by adding 2 M Tris/HCl buffer, pH 8.0. The samples were labelled at 4 °C either by UV or at 480 nm as described above.

### Immunoprecipitation of haemagglutinin (HA) and quantitative analysis of the immunoprecipitation efficiency

Immunoprecipitation of HA was performed using 1:50 dilution of anti-X31/HA antibody as described in [15]. The efficiency of immunoprecipitation was determined using $^{125}$INA-labelled virus. The virus (100 $\mu$g) was labelled with $^{125}$INA in solution by UV irradiation. The radioactively labelled virus was immunoprecipitated according to the protocol [15]. Aliquots of the virus before and after immunoprecipitation were subjected to 12% SDS/PAGE, and quantitative analysis was performed as described earlier. Under the assumption that the HA2 subunit of HA constitutes 20% of the total viral protein and correcting for the difference in the amount of the protein loaded on the gel, 42% of the starting HA was recovered by immunoprecipitation.

### Determination of HA binding to chromaffin cells

The virus (35 $\mu$g) was incubated with DiO and unbound dye was removed by passing the virus through a PD-10 (Pharmacia) desalting column followed by centrifugation through a 10% sucrose gradient at 100000 ***g*** for 1 h at 4 °C. The amount of fluorescence associated with the virus was determined in a Spex fluorimeter after solubilization of the virus in 0.1% Triton X-100. Fluorescently labelled virus was bound to chromaffin cells at pH 7.4, the cells were washed and the fluorescence associated with the cells was measured as described above. It was found that 0.312 $\mu$g (5%) of the total amount of the virus added was bound to the cells.

### Labelling of annexin VII, PKC and BSA

Recombinant annexin VII (15 $\mu$g) [23], PKC (1 $\mu$g) and BSA (5 $\mu$g) in PBS were incubated with $^{125}$INA–DiO containing chromaffin cells or isolated chromaffin granules under conditions specified for each experiment. The samples were labelled either by UV or 480 nm light as described above. Soluble proteins were separated from membrane fractions by centrifugation at 10000 ***g*** for 30 min at 4 °C followed by ultracentrifugation of the supernatant in an 'Airfuge' at 148000 ***g*** for 1 h at 4 °C.

### Immunoprecipitation of annexin VII from bovine chromaffin granule membranes

The bovine chromaffin granules were lysed in RIPA buffer [150 mM NaCl/1% (v.v) Nonidet P-40/0.5% deoxycholate/50 mM Tris/HCl, pH 7.5/5 mM EGTA], and the lysate was collected by centrifugation at 10000 ***g*** for 2 min. Polyclonal anti-annexin VII antibody (50% ammonium sulphate cut) was added to the supernatant in 1:100 dilution and incubated overnight at 4 °C. Protein-G–Sepharose (10%, v/v) was added to the mixture and incubated for another 5 h at 4 °C. The protein-G–Sepharose was washed 5 times with 5 mM Tris/HCl, pH 7.5. The sample was solubilized in SDS/PAGE sample buffer, boiled for 5 min, and subjected to 10% SDS/PAGE.

### Labelling of band 3 from human erythrocyte membrane

Fresh human erythrocyte packed cells (100 $\mu$l; 60% haematocrit) were washed in PBS and resuspended in 1 ml of diluent C (Sigma). The erythrocytes were labelled with DiO as described above for chromaffin cells. After the removal of the un-incorporated DiO, the cells were resuspended in 5 mM phosphate buffer, pH 8.0, and incubated on ice for 15 min. The washing was repeated until the membrane pellet was free of haemoglobin. $^{125}$INA (5 $\mu$Ci) was added to a 500 $\mu$l suspension of red-cell membranes (white ghosts), and the membranes were washed and irradiated at 480 nm for photosensitized labelling. The erythrocyte membrane proteins (35 $\mu$g/lane) were separated by SDS/PAGE followed by PhosphorImager-enhanced autoradiography. In our previous studies with $^{125}$INA-labelled erythrocytes, we identified the 100 kDa band 3 as the only labelled protein of this size [24]. Quantitative analysis was performed as described earlier. The amount of the protein in the band was calculated by assuming that band 3 constitutes 30% by weight of total erythrocyte-membrane protein [25].

### Labelling of gp-170 with $^{125}$INA

Multidrug-resistant human carcinoma KB-V1 cells, cultured and selected as described in [26], were suspended in PBS at a density $1 \times 10^6$ cells/ml and washed twice. The cells were loaded with $^{125}$INA–DiO as described above for chromaffin cells and irradiated with 480 nm light for photosensitized labelling of membrane proteins. Cells were washed, lysed in lysis buffer as described above for chromaffin cells, and subjected to SDS/PAGE followed by autoradiography. In previous studies on the $^{125}$INA-labelled KB-V1 cells, we identified gp-170 as the only labelled membrane protein of this size [13]. Quantitative analysis of labelled gp-170 was performed as described earlier. The amount of the protein was calculated assuming that gp-170 constitutes 1% of total membrane proteins by weight [27].

### Labelling of the dopamine transporter from chromaffin granules

Isolated chromaffin granules were prepared from bovine adrenal glands as described previously [22]. The granules were labelled with $^{125}$INA, and the dopamine transporter was purified to homogeneity by Dr. S. Shuldiner (Hebrew University Hadassah Medical School, Jerusalem, Israel) as described previously [28]. Purification of dopamine transporter was done in the framework of a separate collaborative study (Y. Raviv, S. Schuldiner and M. A. Levine, unpublished work). The INA:protein ratio data were collected expressly for the presentation in this study. The radioactivity of a purified dopamine transporter was determined as described earlier.

## RESULTS

### Identification of GST in the chromaffin cell cytosol as a protein that penetrates reversibly into the membrane lipid bilayer

Photosensitized labelling was applied *in situ* to examine chromaffin cells for proteins in the cytosol that were able to interact reversibly with the lipid phase of the membrane.

Bovine chromaffin cells were loaded with $^{125}$INA and DiO and irradiated at 480 nm. The membrane and the cytosolic fractions were separated and tested for the presence of labelled proteins (Figure 1). As expected, many integral membrane proteins that sedimented with the membrane fraction were labelled (Figure 1, compare lanes A1 and B3). Examination of the chromaffin cell cytosol, however, revealed only one labelled protein of 28 kDa (Figure 1, lanes A2 and B4). The protein was purified from the cytosol to homogeneity, digested with trypsin, and sequences of two peptides were obtained as described in the Experimental section. A sequence homology search identified the 28 kDa protein as a bovine alpha-family GST (Figure 2). DiO distribution under physiological conditions is confined exclusively to the plasma membrane of chromaffin cells, as observed by the confocal image of DiO fluorescence (results not shown). These

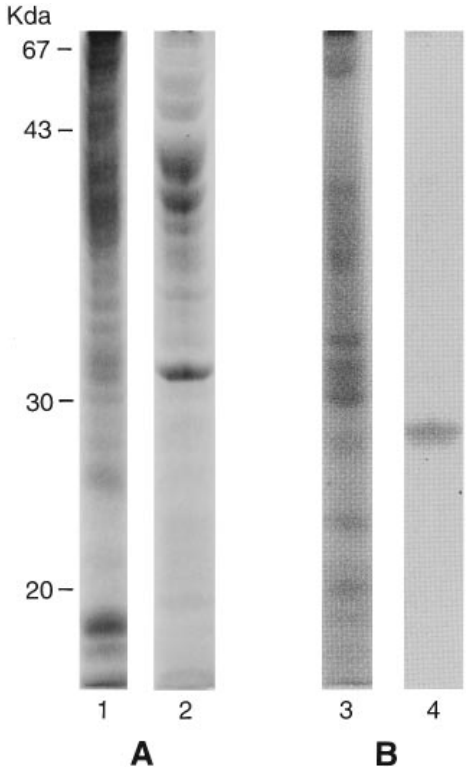


**Figure 1 DiO-induced photosensitized labelling of chromaffin cell proteins**

$^{125}$INA + DiO chromaffin cells were irradiated at 480 nm, and the cytosolic and membrane fractions were separated by centrifugation. Samples were subjected to SDS/PAGE, and radiolabelled proteins were visualized by PhosphorImager-enhanced autoradiography. The images were recorded in the 10–200 colour range. (**A**) Coomassie Brilliant Blue staining; (**B**) autoradiography. Lanes 1 and 3, membrane fraction; lanes 2 and 4, cytosolic fraction.

data suggest that labelling of GST by photosensitization results primarily from its interaction with the chromaffin cell plasma membrane, although interaction with other intracellular compartments cannot be excluded. To test whether GST interacts with chromaffin granules as well, isolated chromaffin granules were loaded with $^{125}$INA and DiO, mixed with fresh chromaffin cell cytosol, and irradiated by visible light. GST was again the only protein in the cytosol that became labelled (Figure 3, lanes A2 and B4). Diffusion of $^{125}$INA from the lipid bilayer into hydrophobic sites on the protein may account for the labelling observed. To examine this possibility, cytosolic fraction was incubated with $^{125}$INA–DiO loaded chromaffin granules, the membrane fraction was removed by sedimentation, and the cytosol was irradiated with UV or visible light. GST became labelled under these conditions *only with UV light* by direct activation of $^{125}$INA molecules that diffused from the cell membrane to the surface of the protein (Figure 3, lane B6). DiO did not diffuse from the membrane to GST, since irradiation of the isolated cytosol with *visible light* in the absence of membranes failed to induce radiolabelling of the protein (Figure 3, lane B5). The interaction of GST with biological membranes required the protein to be in its native conformation. Heating of the cytosolic fraction before the incubation with chromaffin granules containing $^{125}$INA completely abolished labelling of GST (results not shown).

In order to establish conclusively the mechanism of GST–membrane interaction, we tested site-directed photosensitization

| | | |
|---|---|---|
| 28kDa protein | SHGQDYLVGNK | VSNIPAV |
| Glutathione S-transferase A1-1 [Homo sapiens] | SHGQDYLVGNK | ISNLPTV |

**Figure 2 Sequence homology between two peptides obtained from 28 kDa protein and human alpha GST A1–1**

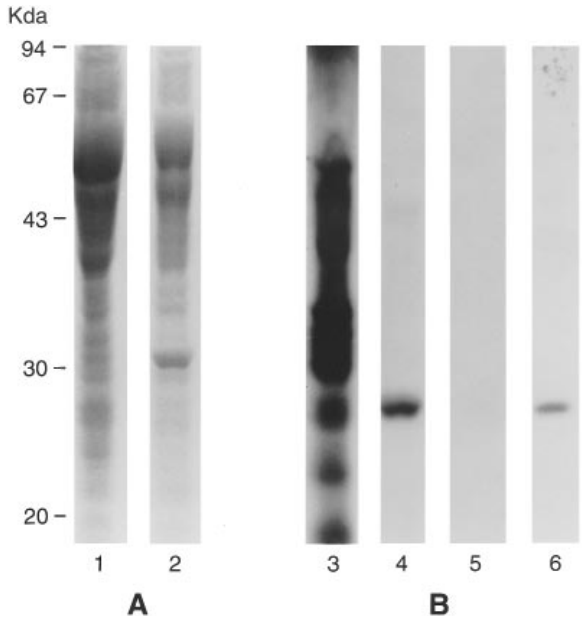


**Figure 3 Labelling of chromaffin cell cytosol upon interaction with $^{125}$INA + DiO chromaffin granules**

Chromaffin cell cytosol was incubated with $^{125}$INA + DiO chromaffin granules at room temperature for 10 min and irradiated at 480 nm. Membrane and cytosolic fractions were separated by centrifugation in an Eppendorf centrifuge (Beckman) at 14000 ***g*** for 30 min. The cytosolic fraction was further centrifuged in an 'Airfuge' at 148000 ***g*** for 30 min. Where indicated, the separation of membrane and cytosolic fractions was performed before irradiation by UV or at 480 nm. The fractions were subjected to 12% SDS/PAGE followed by autoradiography. (**A**) Coomassie Brilliant Blue staining; (**B**) autoradiography of the gels. Lanes 1 and 3, chromaffin granule membranes; lanes 2 and 4, cytosolic fraction irradiated at 480 nm in the presence of chromaffin granules; lane 5, cytosolic fraction irradiated at 480 nm after removal of chromaffin granules; lane 6, cytosolic fraction irradiated with UV light after removal of chromaffin granules.

for its selectivity towards the lipid phase of the membrane. We then compared the penetrating ability of GST with known integral membrane and soluble proteins using the $^{125}$INA : protein ratio as a quantitative measure.

### Labelling of the influenza virus HA in the chromaffin cells is selective towards the membrane lipid bilayer

We used influenza virus fusion protein, HA, as a defined biological experimental model to test the selectivity of DiO-directed photosensitized labelling towards the lipid phase of the membrane. It has been recently shown that, in human erythrocytes, $^{125}$INA labelling of HA occurs only under fusogenic conditions [15]. We wished to determine whether the selectivity of the labelling towards the lipid phase of the membrane is a general phenomenon and can thus be applied to studies in bovine chromaffin cells.

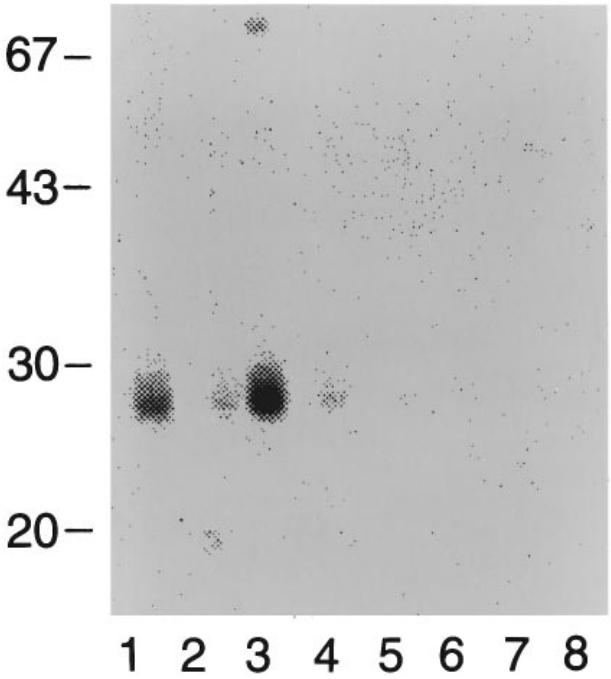


**Figure 4 Labelling of HA2 by $^{125}$INA–DiO-loaded chromaffin cells**

The virus (100 $\mu$g) was bound to $^{125}$INA–DiO chromaffin cells. The samples were either left at pH 7.4 (lanes 2 and 4) or exposed to pH 5.0 (lanes 1 and 3). The samples were reneutralized and irradiated by UV (lanes 1 and 2) or 480 nm (lanes 3–8). HA was immunoprecipitated and separated by 12% SDS/PAGE followed by PhosphorImager enhanced autoradiography. The images were analysed at 7–200 colour range. Controls: without DiO, lanes 5 (pH 5.0) and 6 (pH 7.4); without the virus, lanes 7(pH 5.0) and 8 (pH 7.4).

Insertion of the influenza virus HA into the chromaffin cell membrane caused HA labelling by $^{125}$INA activated both by UV and by energy transfer from DiO (Figure 4, lanes 1 and 3). The labelling of HA was strictly dependent on fusogenic conditions: acidification caused strong $^{125}$INA labelling of HA2 (Figure 4, compare lanes 1 and 2, lanes 3 and 4; see also Table 1). The labelling was specific to the fusion peptide; only the 28 kDa HA2 chain of HA, which contained the fusion peptide, was labelled. No labelling was detected in the 55 kDa HA1 chain, which was also immunoprecipitated by the anti-HA antibody. Quantitative analysis of the labelling of HA2 revealed that upon fusion of the viral envelope with the chromaffin cell membrane (pH 5.0), every molecule of HA incorporates at least one molecule of $^{125}$INA (Table 1). In contrast, mere binding of the virus to the cell membrane (pH 7.4) results in the labelling of only 2–3% of HA molecules.

### Quantitative analysis of the labelling of integral membrane proteins

We wished to develop a quantitative criterion with which to characterize integral membrane proteins and distinguish them from soluble proteins that interact with the membrane peripherally. Selected integral membrane proteins (human erythrocyte anion-transporter band 3, multidrug transporter gp-170 from drug-resistant KB carcinoma cells, dopamine transporter from chromaffin granules and fusion-competent influenza HA) were photolabelled with $^{125}$INA *in situ*, isolated subsequently, and the $^{125}$INA:protein molar ratio was calculated for each protein. The values obtained are listed in Table 1.

### Labelling of soluble proteins that interact with membranes

Three soluble proteins (annexin VII, PKC and BSA), which are known to interact with biological membranes under specific conditions, were examined by photosensitized labelling for their ability to penetrate into the lipid bilayer. The $^{125}$INA:protein molar ratio was calculated as for the integral membrane proteins, and the values obtained were compared with those observed for integral proteins (Table 1).

#### Annexin VII

Annexins bind reversibly to phospholipid bilayers and biological membranes in the presence of $Ca^{2+}$ [29]. They have high affinity for acidic phospholipids, such as phosphatidylserine and phosphatidylinositol, which are displayed on the cytoplasmic (inner) surface of the cell plasma membrane and on the cytoplasmic (outer) surface of secretory granule membranes [29]. Isolated bovine chromaffin granules were loaded with $^{125}$INA and DiO and incubated with recombinant human annexin VII in the presence of 1 mM $Ca^{2+}$. Under these conditions, annexin VII became completely associated with the chromaffin granules (Figure 5, compare lanes A2 and A3). The granules were irradiated to induce photosensitized labelling of integral membrane proteins and subsequently treated with 5 mM EGTA, which caused the dissociation of annexin VII from the granule membrane into the medium (Figure 5, lane A4). The released protein was examined for $^{125}$INA labelling by autoradiography, and the molar $^{125}$INA:annexin VII ratio was measured. The faint labelling observed in the protein (Figure 5, lane B8) represents a $^{125}$INA:annexin VII value of 0.002 (mol/mol) (Table 1). It was also obtained in the absence of $Ca^{2+}$ (5 mM EGTA in the medium) (Figure 5, lane B6). A small fraction of the annexin VII molecules remained associated with the granule membrane after EGTA extraction. This residual annexin VII was isolated from the membranes by immunoprecipitation. No labelling was detected on the EGTA-resistant fraction of the protein (Figure 5, lanes C10 and C11).

#### PKC

In the presence of micromolar concentrations of $Ca^{2+}$, $Ca^{2+}$-dependent isoforms of PKC bind to membranes, where they are activated by diacylglycerol or phorbol esters [30]. Chromaffin granules have been shown to constitute a target for the translocation, binding and activation of intracellular PKC from adrenal medulla in a $Ca^{2+}$ and/or phorbol ester-dependent manner [31]. Chromaffin granules loaded with $^{125}$INA and DiO were used to examine the interaction of rat brain PKC with the membrane bilayer in a way similar to the one described for annexin VII. Soluble PKC was bound to the granules in the presence of either 0.5 $\mu$M PMA and/or 50 $\mu$M $Ca^{2+}$, and the samples were irradiated to induce photosensitized labelling of integral membrane proteins. PKC was isolated from the granules by incubation with 5 mM EGTA, and the isolated PKC was analysed for $^{125}$INA labelling. No detectable labelling of PKC was observed under these conditions (results not shown).

#### BSA

Serum albumins interact with cell membranes in a process resulting in the exchange of fatty acids and other hydrophobic molecules between the cell and specific hydrophobic binding sites

**Table 1 Quantitative analysis of photosensitization-induced incorporation of $^{125}$INA into integral membrane proteins versus soluble proteins**

Notes: [a]The molar ratio, $R$ ($^{125}$INA:protein), was calculated as:

$R = [(A)/(B \times 0.2)]$

where $A$ is measured radioactivity incorporated into the protein ($\mu$Ci), $B$ is amount of the protein (mol) and 0.2 is the specific radioactivity of $^{125}$INA (mCi/$\mu$mol). [b]The molar ratio $R^*$ ($^{125}$INA:HA) was calculated as:

$R^* = [(A)/(kB \times 0.2)]$

where $A$ is measured radioactivity incorporated in the immunoprecipitated HA ($\mu$Ci), $B$ is the amount of HA bound (mol) and $k$ is the efficiency of immunoprecipitation. $A$, $B$ and $k$ were determined as described in Experimental section. [c]The molar ratio $R$ ($^{125}$INA:P28) was determined as in note a except the amount of the protein ($B$) was determined by densitometric analysis as described in Experimental section.

| Integral protein | $^{125}$INA:protein | Soluble protein | $^{125}$INA:protein |
|---|---|---|---|
| Band 3 | 1[a] | Annexin VII | 0.002 |
| Influenza virus HA (pH 5.0) | 2[b] | Influenza virus HA (pH 7.4) | 0.023 |
| gp-170 | 8 | BSA | 0.002 |
| Dopamine transporter | 2 | PKC | 0.000 |
| | | alpha-GST | 1[c] |

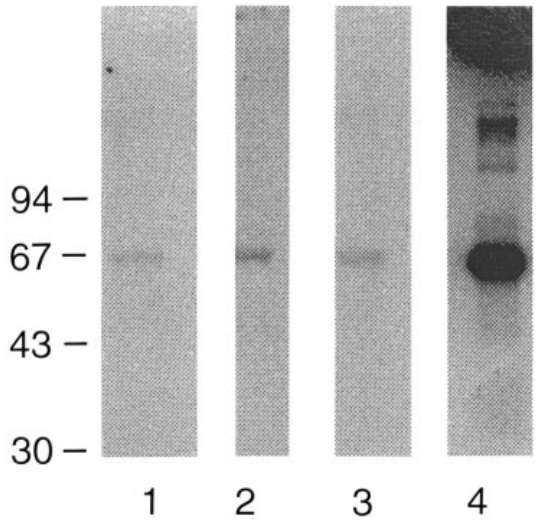


**Figure 6 Labelling of BSA upon interaction with $^{125}$INA + DiO chromaffin cells and granules**

BSA (5 $\mu$g) was incubated with $^{125}$INA + DiO chromaffin cells or chromaffin granules for 10 min at room temperature. The samples were irradiated at 480 nm. After irradiation, BSA was separated from chromaffin cells or granules by centrifugation, and the samples were subjected to 10% SDS/PAGE followed by PhosphorImager-enhanced autoradiography. Where indicated, the separation was performed first, followed by the irradiation with UV light. The radioactive images were recorded in the 12–150 colour range. Lane 1, BSA irradiated at 480 nm in the presence of chromaffin cells; lane 2, BSA irradiated at 480 nm in the presence of chromaffin granules; lane 3, BSA irradiated with UV light after removal of chromaffin cells; lane 4, BSA labelled in solution.

on the protein [32]. In our study, photosensitized labelling was used to examine the interaction of BSA with the membrane bilayer of both chromaffin cells and isolated granules. The experimental protocol was similar to the one described for annexin VII and PKC, except that no specific activators were necessary. When BSA was added to $^{125}$INA–DiO containing chromaffin cells, the protein became labelled weakly by photosensitized $^{125}$INA (Figure 6, lane 1). These results did not depend on whether BSA was complexed with fatty acids or not (results not shown). The molar $^{125}$INA:BSA ratio was found to be 0.002, remarkably similar to the value obtained for annexin VII (Table 1). A similar result was obtained when chromaffin granules were used instead of chromaffin cells (Figure 6, lane 2). Interaction of BSA with chromaffin cells and granules was accompanied by the diffusion of $^{125}$INA from the granule membrane to the surface of the protein (Figure 6, lane 3).

### Quantitative analysis of $^{125}$INA-labelled $\alpha$-GST

The molar $^{125}$INA:protein ratio for bovine $\alpha$-GST was determined as described in the Experimental section, and it was

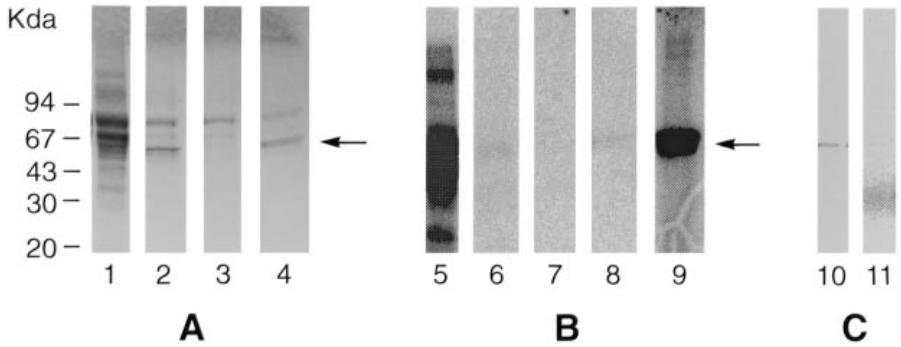


**Figure 5 Labelling of annexin VII upon interaction with $^{125}$INA–DiO-loaded chromaffin granules**

Recombinant human annexin VII (15 $\mu$g) was incubated with chromaffin granules loaded with $^{125}$INA + DiO in the presence or absence (5 mM EGTA) of $Ca^{2+}$. The mixture was irradiated at 480 nm, and the granules were sedimented by centrifugation at 10000 ***g*** for 15 min. Different fractions were subjected to the SDS/PAGE followed by the PhosphorImager-enhanced autoradiography. The images were recorded in the 5–150 colour range. (**A**) Coomassie Brilliant Blue staining of the gel; (**B**) PhosphorImager-enhanced autoradiography. Lanes 1 and 5, sedimented chromaffin granules; lanes 2 and 6, supernatant of annexin VII incubated with granules in the absence of $Ca^{2+}$ (5 mM EGTA); lanes 3 and 7, as in 2 and 6, only in the presence of 1 mM $Ca^{2+}$; lanes 4 and 8, annexin VII prebound to the granules in the presence of 1 mM $Ca^{2+}$ and then recovered from the membrane by EGTA extraction; lane 9, annexin VII labelled in solution. (**C**) Western-blot analysis of immunoprecipitated annexin VII: lane 10, immunostaining; lane 11, autoradiography.

found to be 1, compatible with the values obtained for integral membrane proteins (Table 1).

## DISCUSSION

In this work we introduce site-directed photosensitization to study interactions of proteins with biological membranes *in situ*, and especially those that involve the transition of proteins from soluble to membrane-bound. We identify $\alpha$-GST as the only labelled soluble protein in bovine chromaffin cell cytosol using a method that was designed to label exclusively integral membrane proteins. A plausible and attractive interpretation of this result is that GST penetrates transiently into the lipid phase of the cell plasma membrane and is subsequently released back into the cytosol. Such an explanation will not be entirely surprising considering the biological role of this enzyme. GSTs belong to a family of phase-II detoxifying enzymes. Detoxification is achieved by conjugation of glutathione with electrophilic substrates, many of them hydrophobic xenobiotics, which partition into membrane compartments [33]. Access of GST to both hydrophobic xenobiotics and glutathione may require fast transitions of the enzyme between the membrane phase and the aqueous cytosol. In order to validate this hypothesis and exclude the possibility of random non-selective labelling of cytosolic proteins, we carried out a thorough examination of the photolabelling method employed. The examination included testing the selectivity of labelling toward the hydrophobic phase of the membrane in a defined biological experimental system, and establishing a quantitative criterion by which to distinguish between proteins that penetrate into the lipid bilayer and those that do not.

Fusion of influenza virus envelope with the chromaffin cell membrane was used as a model system to test the selectivity of $^{125}$INA labelling towards the lipid phase of the membrane. The interaction and fusion of the virus with the target cell membrane is facilitated by a trimer of the viral envelope glycoprotein HA [34,35]. Binding of the virus to the cell occurs at neutral pH. Exposure of the virus to acidic pH (5.0) triggers a multistep process which starts with the insertion of a hydrophobic peptide from the HA2 subunit of HA into the host cell membrane, and ends with the fusion between the viral envelope and the cell membrane [36]. The selectivity of photosensitized labelling towards the lipid bilayer of the membrane is demonstrated in this system by the observation that HA2 (which bears the fusion peptide) is strongly labelled only under the conditions that trigger its insertion into the cell plasma membrane (acidic pH). The labelling is limited to HA2 and not to the HA1 subunit. Some incorporation of $^{125}$INA into HA2 occurs in the binding step at neutral pH, and it amounts to 2% of the labelling after insertion of the protein (Table 1). In this system, HA2 labelling increases by two orders of magnitude after its transition from a peripherally bound viral polypeptide to a membrane-integrated one. The observed changes in the labelling most likely represent the changes in the distance between the viral envelope and the cell membrane during binding and fusion. Energy transfer processes induced by photoexcitation of carbocyanine derivatives (DiO) are based on electron transfer [37,38]. Studies in monomolecular layers have demonstrated that photoinduced electron transfer to an acceptor molecule can occur from a distance, across a non-conducting barrier, by quantum-mechanical electron tunnelling [39,40]. The labelling observed on HA2 in the binding step may be the result of electron tunnelling from the DiO in the cell membrane to $^{125}$INA, which diffused into the viral envelope. The sharp decrease in the labelling with the increase of distance between HA2 and cell membrane is consistent with the exponential decrease of electron-transfer efficiency with distance predicted by the quantum-mechanical tunnelling theory [38]. This explanation assumes that $^{125}$INA concentration at all distances considered is not a limiting factor. Labelling of HA2 by $^{125}$INA radicals that originate in the cell membrane and diffuse to the viral envelope is unlikely because of the presence of 20 mM glutathione in the medium, and because the labelling was selective towards HA2 and not HA1. Hence, the large difference in the extent of $^{125}$INA labelling of HA2 before and after fusion obtained by site-directed photosensitization provides a good quantitative criterion for distinguishing between integral membrane- and peripherally bound proteins. This is further supported by the observation that the extent of $^{125}$INA incorporation in three different integral membrane proteins is three orders of magnitude larger than the values obtained in three different soluble proteins that interact with the membrane. The results listed in Table 1 indicate that, for the integral membrane proteins tested, the labelling reaction in the lipid phase of the membrane is efficient to the extent that, on average, at least 1 molecule of $^{125}$INA is incorporated into every molecule of protein (band 3) and it can reach as many as 8 molecules of $^{125}$INA per protein molecule (gp-170). These results indicate that $^{125}$INA incorporation into the integral membrane proteins is not necessarily proportional to the number of transmembrane segments present in the protein. The availability of sites with variable chemical reactivity towards $^{125}$INA photoadducts, as well as the accessibility of these sites for labelling, is determined by the topological organization of the proteins in the membrane and may account for the variability of the labelling observed. Therefore, some integral membrane proteins may display $^{125}$INA:protein values less than 1 or greater than 8. It is unlikely, however, that these factors alone can explain labelling of annexin VII and BSA that was three orders of magnitude lower. Efficient labelling of annexin VII and BSA was obtained when it was performed nonspecifically in solution in the absence of membranes (Figure 5, lane 9 and Figure 6, lane 4), indicating an abundance of potential reactive sites for INA in these proteins. The $^{125}$INA:protein value of 0.002 most probably indicates that these proteins interact with the membrane at a distance from the lipid bilayer. The same conclusion can be made about PKC, which showed no appreciable labelling. This conclusion is further supported by studies on $Ca^{2+}$ and PMA-induced association of PKC $\beta$II with phospholipid membranes [41].

The only $^{125}$INA-labelled protein observed in bovine chromaffin cell cytosol is $\alpha$-GST. Although recovered in the cytosol, this GST displays the labelling characteristics of an integral membrane protein with an $^{125}$INA:protein value of 1. In this respect, the difference between the labelling of GST and BSA is very interesting. Both proteins bind a wide array of hydrophobic substances, including $^{125}$INA, and both proteins interact with either inner (GST) or outer (BSA) membrane leaflets, yet only GST penetrates into the hydrophobic phase of the lipid bilayer. This result suggests that the interaction of $\alpha$-GST with the membrane transiently puts some of its domains in sufficient proximity to the photosensitizing process in the bilayer to be tagged by the radioactive probe. This proximity may be a result of a direct interaction with the lipid domain of the membrane, where hydrophobic substrates may reside, or it can be facilitated by receptor proteins on the cell membrane. Such putative receptors may be transport proteins which facilitate efflux of conjugates of GST substrates with glutathione outside the cell for further processing, or transporters that carry GST ligands into the cell [42,43]. A multi-drug resistance protein has been recently identified as a glutathione S-conjugate export pump in erythrocytes [44].

From the methodological point of view, the present study shows that targeted photoactivation of $^{125}$INA molecules in the membrane lipid bilayer is a fast process that can radioactively tag proteins transiently introduced in this compartment. This radioactive signal creates a 'memory' of past insertion into the membrane that can later be retrieved and followed for further study or separation.

Since this paper was originally submitted we have come across a bovine GST sequence, accession number U49179, submitted directly to the GenBank Nucleotide Sequence database, which shows 100 % homology with the present enzyme.

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