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Selective Radiolabeling of Cell Surface Proteins to a High Specific Activity†

James A. Thompson, Alice L. Lau, and Dennis D. Cunningham*
Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92717
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ABSTRACT: A procedure was developed for selective radiolabeling of membrane proteins on cells to higher specific activities than possible with available techniques. Cell surface amino groups were derivitized with ^{125}I-(hydroxyphenyl)propionyl groups via ^{125}I-sulfosuccinimidyl (hydroxyphenyl)propionate (^{125}I-sulfo-SHPP). This reagent preferentially labeled membrane proteins exposed at the cell surface of erythrocytes as assessed by the degree of radiolabel incorporation into erythrocyte ghost proteins and hemoglobin. Comparison with the lactoperoxidase–[^{125}I]iodide labeling technique revealed that ^{125}I-sulfo-SHPP labeled cell surface proteins to a much higher specific activity and hemoglobin to a much lower specific activity. Additionally, this reagent was used for selective radiolabeling of membrane proteins on the cytoplasmic face of the plasma membrane by blocking exofacial amino groups with uniodinated sulfo-SHPP, lysing the cells, and then incubating them with ^{125}I-sulfo-SHPP. Exclusive labeling of either side of the plasma membrane was demonstrated by the labeling of some marker proteins with well-defined spacial orientations on erythrocytes. Transmembrane proteins such as the epidermal growth factor receptor on cultured cells could also be labeled differentially from either side of the plasma membrane.

In many studies where it is important to identify surface membrane proteins in low abundance, it is critical to radiolabel them to a high specific activity under conditions that label cytoplasmic proteins only minimally (Moss & Cunningham, 1981; Phillips & Agin, 1974). Several procedures have been developed to label surface membrane proteins, although each has certain limitations. Metabolic labeling of cultured cells with [^{3}H]fucose (Atkinson & Summers, 1974) or [^{3}H]-glucosamine (Liau & Horowitz, 1982) results in the labeling of glycoproteins of both the plasma membrane and also intracellular membranes. In addition, these procedures take long periods of time and results in labeled proteins of relatively low specific activity. Other methods involve the oxidation of membrane glycoproteins with galactose oxidase (Baumann & Doyle, 1978) or periodate (Steck & Dawson, 1974) followed by reduction with sodium [^{3}H]borohydride. However, proteins labeled with tritium very poorly expose X-ray film during autoradiography because of the low energy of this isotope (Bonner & Laskey, 1974). The most common method for labeling cell surface proteins with a high-energy isotope involves the generation of [^{125}I]iodine by incubating cells with [^{125}I]iodide plus hydrogen peroxide (Phillips & Morrison, 1970; Morrison, 1974, 1980) or with [^{125}I]iodide plus lactoperoxidase and glucose oxidase (Hubbard & Cohn, 1975). However, this procedure does not label membrane proteins to a high specific activity; only 0.1% of the available tyrosines become labeled (Hubbard & Cohn, 1975). Chloroglycoluril has also been used to label membrane proteins on cells with ^{125}I; however, there is significant labeling of cytoplasmic proteins when conditions are adjusted to achieve very high specific activities of membrane proteins (Markwell & Fox, 1978). There are other membrane-impermeant labeling agents that can be iodinated, but they are low in reactivity with membrane proteins at a neutral pH (Cabantchik & Rothstein, 1974; Berg, 1969).

In the present study, a procedure was developed to radiolabel membrane proteins to a high specific activity with a high-energy isotope with little labeling of cytoplasmic proteins. This procedure utilizes a water-soluble form of the iodinated Bolton–Hunter reagent, ^{125}I-sulfosuccinimidyl (hydroxyphenyl)propionate (^{125}I-sulfo-SHPP).[1] This reagent retains the highly reactive amino-labeling properties of the Bolton–Hunter reagent (Bolton & Hunter, 1973) but is rendered membrane impermeable by sulfation. Experiments were conducted with erythrocytes to evaluate the degree of surface membrane vs. cytoplasmic labeling. Clean cytoplasmic and membrane fractions can easily be obtained from erythrocytes. Also, erythrocytes have several protein markers that have been well characterized that sharply delineate the cytoplasmic space as well as the inner and outer faces of the plasma membrane

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* Correspondence should be addressed to this author.

[1] Abbreviations: sulfo-SHPP, sulfosuccinimidyl (hydroxyphenyl)-propionate; HF, human foreskin; CHEF, Chinese hamster embryonic fibroblast; EDTA, (ethylenedinitrilo)tetraacetic acid; PBS, phosphate-buffered saline; D-PBS, Dulbecco's modified phosphate-buffered saline; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGF, epidermal growth factor; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; kDa, kilodalton(s); TPCK, tosylphenylalanine chloromethyl ketone.

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[see Marchesi et al. (1976) for a review].

Further studies showed that certain properties of sulfo-SHPP could be exploited to examine the spacial orientation of proteins in the surface membrane. Since proteins at the cell surface could be reacted with nonlabeled sulfo-SHPP, it was possible to block exofacial amino groups, lyse the cells, and then incubate with ^{125}I-sulfo-SHPP for specific labeling of the cytoplasmic face of membrane proteins. This was demonstrated both with erythrocytes and with cultured fibroblast-like cells.

Materials and Methods

Materials. Carrier-free Na^{125}I was purchased from New England Nuclear (Boston, MA). Sulfo-SHPP was obtained from Pierce (Rockford, IL). A monoclonal antibody directed against the EGF receptor was obtained from Dr. G. Carpenter, Vanderbilt University. Electrophoretic reagents were obtained from Bio-Rad (Richman, CA). Cell culture supplies were from GIBCO (Grand Island, NY). Lactoperoxidase and glucose oxidase were from Calbiochem (San Diego, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. HF cells and A431 cells were grown and maintained in 100-mm tissue culture dishes (Corning) in Dulbecco's modified Eagle's medium containing 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, and 5% fetal calf serum (Baker et al., 1980). CHEF cells were maintained as previously described (Low et al., 1985). Cells were passaged weekly at a split ratio of 1:5. At confluence (2×10^6 cells/plate), cultures were rinsed with serum-free medium and incubated in this medium for 2 days before being labeled.

Tissue culture cells were lysed by a modification of the method of Barber and Jamieson (1970). Briefly, cells in 100-mm tissue culture dishes were washed with D-PBS and allowed to equilibrate in 5 mL of PBS containing 50% glycerol for 10 min at 4 °C. The glycerol solution was removed, and cells were lysed by the addition of 5 mL of cold 10 mM HEPES, pH 7.4, containing 1 mM $MgCl_2$. Under these conditions, no whole cells could be found by phase-contrast microscopy.

Radioiodination of Sulfo-SHPP. Sulfo-SHPP was iodinated with ^{125}I by a modification of the method described by Bolton and Hunter (1973). A 0.2 mg/mL stock solution of sulfo-SHPP in dimethyl sulfoxide was prepared; it should be used no longer than 2 weeks after preparation. One to twenty microliters of this stock was placed in a conical microfuge tube, and the following reagents were added quickly and in succession from freshly made stocks: 1–20 μL of 100 mCi/mL Na^{125}I, 10 μL of chloramine-T (5 mg/mL in 0.5 M sodium phosphate, pH 7.5), 100 μL of hydroxyphenylacetic acid (1 mg/mL in water), and 10 μL of sodium metabisulfate (12 mg/mL in 0.05 M sodium phosphate, pH 7.5).

Preparation of Erythrocytes and Erythrocyte Ghosts. Fresh whole human blood was collected and diluted with a 10-fold volume of 1 mM EDTA in PBS. Erythrocytes were collected by centrifugation at 100*g* for 5 min in a clinical centrifuge and washed 3 times in the same buffer. The number of erythrocytes was quantitated by a Coulter electronic particle counter.

Erythrocyte ghosts were prepared by lysis in a 10-fold volume of 1 mM EDTA and 10 mM HEPES, pH 7.4 at 0 °C, containing 1 mM benzamidine hydrochloride, 10 μM chymostatin, 10 μM pepstatin, 10 μM leupeptin, 10 μM antipain, 10 μM aprotinin, and 1 mM phenylmethanesulfonyl fluoride. Membranes were sedimented in a Beckman Microfuge B for 5 min and washed 3 times in the above lysis buffer. Cells labeled with ^{125}I-sulfo-SHPP were lysed in the above lysis buffer containing 1 mg/mL lysine to prevent iodination of cytoplasmic proteins by residual ^{125}I-sulfo-SHPP.

Radioiodination of Erythrocytes. Washed erythrocytes were radiolabeled by direct addition of the above ^{125}I-sulfo-SHPP reaction mixture to erythrocytes in D-PBS. Sulfo-SHPP must be iodinated immediately prior to use to minimize hydrolysis of the succinimide ester. The reaction was typically allowed to proceed for 30 min on ice. The volume of the reaction, number of erythrocytes, and amount of ^{125}I-sulfo-SHPP were varied as indicated in the figure legends. The reaction was terminated by adding 1 mL of 1 mg/mL lysine in D-PBS. All of the above steps were conducted in a fume hood. Cells were collected by centrifugation for 5 min at 100*g* and washed 3 times in the same buffer.

Radioiodination of Erythrocytes with Lactoperoxidase. Erythrocytes were iodinated by using lactoperoxidase and glucose oxidase with Na^{125}I at 22 °C as described by Hubbard and Cohn (1975). The reaction was terminated by dilution of 100 μL of the radioiodination mixture into 1 mL of 10 μg/mL NaI in D-PBS containing 0.1% sodium azide. Cells were collected by centrifugation for 5 min at 100*g* and washed 3 times in the same buffer.

Trichloroacetic Acid Precipitation. Proteins from whole erythrocytes or ghosts were typically solubilized in 100 μL of 1% SDS. To precipitate the proteins, 10 μL was added to 1 mL of cold 10% trichloroacetic acid and incubated on ice for 10 min. Precipitates were collected by filtration on Whatman GF/C filters and washed with 2 mL of cold 10% trichloroacetic acid. ^{125}I was quantitated in a Beckman γ counter.

Blocking of Surface Amino Groups. Amino termini and lysine groups exposed at the cell surface were blocked by incubating intact erythrocytes with 100 μg/mL unlabeled sulfo-SHPP for 20 min at 4 °C. Sulfo-SHPP was added to erythrocytes from a stock solution of 1 mg/mL in water and prepared immediately before use. Ethanol must be avoided because high concentrations can cause lysis of erythrocytes. Erythrocytes with blocked amino groups were then washed once with 1 mL of 1 mg/mL lysine in D-PBS and twice with 1 mL of D-PBS; after each wash, they were collected by centrifugation for 5 min at 100*g* at 4 °C.

Surface amino termini and lysine groups of tissue culture cells were derivatized by the addition of 1–2 mg/mL sulfo-SHPP. More reagent was needed to effectively block the amino groups on cells in culture than was needed for erythrocytes.

Radioiodination of Tissue Culture Cells. Washed tissue culture cells in 100-mm tissue culture dishes were radiolabeled as described for erythrocytes except that ^{125}I-sulfo-SHPP was diluted in 5 mL of D-PBS for each plate. The iodination was terminated by aspiration of the iodination mixture and washing of the cells in the dishes 3 times with 10 mL of D-PBS containing 1 mg/mL lysine.

Selective Extraction of Membrane Proteins. Membrane proteins were selectively extracted into four groups by a modification of the procedure of Thompson and Cunningham (1986). In the present experiments, prior purification of the membrane was not necessary since membrane proteins were specifically labeled with ^{125}I. Briefly, 100-mm dishes of confluent, serum-starved HF cells were rinsed 3 times with 10 mL of PBS. The first group of membrane proteins was extracted from the surface of intact HF cells by a 10-min incubation at 22 °C with 2 mL of 1 mM EDTA, 0.15 M NaCl, and 10 mM HEPES, pH 7.4 (fraction 1). This fraction was removed after cells were scraped from the substratum with a rubber policeman and collected by centrifugation at 100*g*

for 5 min in a conical polypropylene tube. The second and third groups of membrane proteins were obtained by treatment of the EDTA-extracted cell pellet from each dish with 100 μL of 2% Triton X-114 [prepared as described by Bordier (1981)] in 10 mM HEPES, pH 7.4. Insoluble material was removed by centrifugation in a Beckman Airfuge for 20 min. After addition of 30 μL of 4 M NaCl, the Triton X-114 extract was incubated for 15 min at room temperature. This facilitated the separation of the extract into two phases by centrifugation at 22 °C for 30 s in the Beckman Microfuge B. The upper detergent-free phase contained hydrophobic proteins (fraction 3). The final fraction was obtained by suspension of the above Triton X-114 insoluble material in 100 μL of SDS sample buffer (fraction 4). Acetone was added to the first three fractions to a final concentration of 90% to precipitate the proteins. This facilitated a concentration of the samples as well as removal of excess Triton X-114. Acetone precipitates were dried in vacuo before suspension in 100 μL of SDS sample buffer. All samples were centrifuged in the Beckman Airfuge for 20 min immeditely before electrophoresis.

Immunoprecipitation of the EGF Receptor. After radiolabeling of A431 cells, the EGF receptor was solubilized and immunoprecipitated with a monoclonal antibody against the EGF receptor as described by Soderquist and Carpenter (1984). Trypsin treatment of the EGF receptor was carried out after immunoprecipitation as described by Basu et al. (1984).

Gel Electrophoresis and Protein Quantitation. SDS–PAGE was performed by a modification of the method of Laemmli (1970). Typically, protein samples were electrophoresed on 7.5–15% polyacrylamide gradient cells. The 15% polyacrylamide stock contained 30% glycerol (Anderson & Peterson, 1981). SDS was excluded from the stacking buffer, separating gel buffer, and the lower reservoir buffer to enhance resolution of lower molecular weight proteins (Wyckoff et al., 1977). Gels were run at 18-mA constant current.

Gels were double stained with silver and Coomassie Blue according to the method of Dzandu et al. (1985). With this method, proteins can be identified by differential staining as well as by molecular weight.

Protein determination was conducted essentially as described by Nakamura et al. (1985) except that the degree of dye binding was determined by video densitometry as described by Mariash (1983), instead of scanning spots at an optical density of 610 nm.

Kodak XAR-5 X-OMAT film was exposed to dried gels for 4–48 h at 70 °C without the use of an intensification screen. Sharper bands were obtained without the screen.

Results

Labeling of Erythrocyte Proteins with ^{125}I-Sulfo-SHPP. Because of the usefulness of the Bolton–Hunter reagent (SHPP) to label proteins to a high specific activity by covalent coupling to amino termini or lysine groups, this reagent was tested on erythrocytes. However, due to its hydrophobic nature, SHPP permeated the membrane of intact erythrocytes and extensively labeled cytoplasmic proteins (data not shown). Because sulfation of certain succinimide ester cross-linking reagents renders them membrane impermeable (Staros & Kakkad, 1983), a sulfated form of the Bolton–Hunter reagent was also tested for its ability to label erythrocyte proteins.

The data in Figure 1 demonstrate that ^{125}I-sulfo-SHPP can be used to label erythrocyte proteins. The original method described by Bolton and Hunter was used to iodinate sulfo-SHPP with ^{125}I. Unfortunately, it was not feasible to separate residual unreacted iodide from the hydrophilic sulfo-SHPP

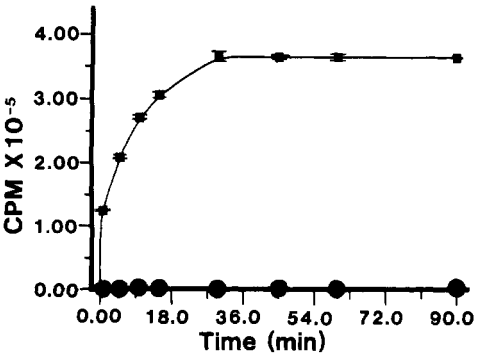

FIGURE 1: Time course of labeling erythrocytes with ^{125}I-sulfo-SHPP. Duplicate aliqots of 5 × 10^8 washed erythrocytes in 1 mL of D-PBS were labeled with 15 μg of ^{125}I-sulfo-SHPP (■) or an identical reaction mixture minus sulfo-SHPP (●) as described under Materials and Methods. 1 mCi of Na^{125}I was used to iodinate 30 μg of sulfo-SHPP. At the indicated times, 10 μL of erythrocytes was removed from the iodination mixtures, quenched with 100 μL of 1 mg/mL lysine in PBS, precipitated with cold 10% trichloroacetic acid, and assayed for radioactivity.

molecule in a nonaqueous solvent. A nonaqueous solvent must be used to prevent hydrolysis of the succinimide ester. Therefore, after iodination, the specific activity of ^{125}I-sulfo-SHPP could not be determined. Nevertheless, after addition of excess hydroxyphenylacetic acid to react with remaining free iodine, the reaction mixture could be added directly to washed erythrocytes. This produced a time-dependent incorporation of ^{125}I into the trichloroacetic acid precipitable material from solubilized erythrocytes as shown in Figure 1. Under these conditions, the incorporation of ^{125}I was complete after 30 min. It is important to point out that radioiodination mixtures lacking sulfo-SHPP produced no detectable incorporation of ^{125}I into erythrocyte trichloroacetic acid precipitable material after addition to washed erythrocytes (Figure 1). This indicated that the iodination of proteins was solely due to the reaction of ^{125}I-sulfo-SHPP with erythrocytes. Although omitting hydroxyphenylacetic acid had no effect on the labeling (data not shown), it was included to prevent volatilization of free $^{125}I_2$. Further characterization of this reaction showed that the pH optimum for the incorporation of ^{125}I-sulfo-SHPP into erythrocytes was 7.4–7.5 (data not shown).

Selective Labeling of Membrane Proteins with ^{125}I-Sulfo-SHPP. The extent to which membrane labeling procedures also led to the labeling of cytoplasmic proteins was evaluated by measuring the labeling of hemoglobin. The amount of hemoglobin labeling was measured after labeling erythrocytes with ^{125}I-sulfo-SHPP as well as with [^{125}I]iodide, lactoperoxidase, glucose oxidase, and glucose, a well-characterized membrane labeling procedure. This permitted a comparison of the two techniques in terms of their ability to selectively label surface membrane proteins. Hemoglobin was a good marker for the degree of cytoplasmic labeling because it represents greater than 95% of erythrocyte cytoplasmic proteins (as determined by video densitometry of stained polyacrylamide gels) and it was readily labeled in lysed erythrocytes by both the ^{125}I-sulfo-SHPP technique (refer to Figure 5) and the lactoperoxidase technique (data not shown). After these points were established, intact erythrocytes were labeled with ^{125}I-sulfo-SHPP or [^{125}I]iodide and lactoperoxidase. Labeled erythrocytes were then lysed and fractionated into cytoplasmic and membrane fractions. Proteins from these fractions were then separated by polyacrylamide gel electrophoresis. Hemoglobin from labeled cells was readily identified on the gels, cut out, and assayed directly for radioactivity. The specific activity of hemoglobin was then determined; the

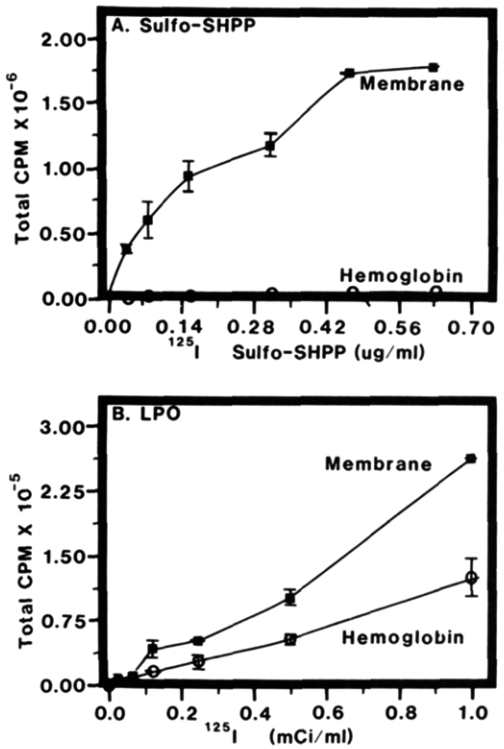

FIGURE 2: Comparison of erythrocytes labeled with either ^{125}I-sulfo-SHPP or Na^{125}I and lactoperoxidase. A total of 3.5×10^7 washed erythrocytes were labeled with various amounts of ^{125}I-sulfo-SHPP on ice for 30 min (panel A) or with various amounts of Na^{125}I with fixed concentrations of lactoperoxidase, glucose oxidase, and glucose at 22 °C (panel B) as described under Materials and Methods. Erythrocyte ghosts and cytoplasmic fractions were prepared as described under Materials and Methods. Proteins from erythrocyte ghosts were solubilized in 1% SDS, precipitated with cold 10% trichloroacetic acid, and assayed for radioactivity (■). Cytoplasmic fractions were electrophoreses on a 7.5–15% polyacrylamide gel. Hemoglobin (○) was exised from dried polyacrylamide gels as well as resolved bands and assayed for radioactivity.

amount of cytoplasmic protein loaded on a gel gave reasonable estimates of the amount of hemoglobin.

The analysis was conducted for erythrocytes labeled over a wide range of specific activities with ^{125}I-sulfo-SHPP or with [^{125}I]iodide plus lactoperoxidase. As shown in Figure 2A, addition of increasing concentrations of ^{125}I-sulfo-SHPP to erythrocytes increased the trichloroacetic acid precipitable radioactivity in the membrane fraction without significantly affecting the amount of ^{125}I in hemoglobin. The amount of ^{125}I in the membrane fraction leveled off at higher concentrations of ^{125}I-sulfo-SHPP, indicating that there was a saturation of reactive sites on the erythrocytes. In contrast, as shown in Figure 2b, increasing concentrations of [^{125}I]iodide during lactoeroxidase-catalyzed iodination of erythrocytes resulted in increasing trichloroacetic acid precipitable radioactivity in both the membrane and hemoglobin fractions. The increased labeling of hemoglobin at higher concentrations of [^{125}I]iodine (Figure 2B) is probably due to permeabilization of the membrane by [^{125}I]iodine (Morrison, 1980). Lactoperoxidase, glucose oxidase, and glucose were held constant at concentrations suggested by other investigators (Hubbard & Cohn, 1975). The conditions of Figure 2 were chosen to achieve high specific activity labeling of membrane proteins with both procedures. It is important to emphasize that with the lactoperoxidase technique, the selectivity for labeling of membrane proteins can be increased over that obtained in Figure 2B by lowering the concentration of [^{125}I]iodide. However, this also decreases the specific activity of the membrane proteins.

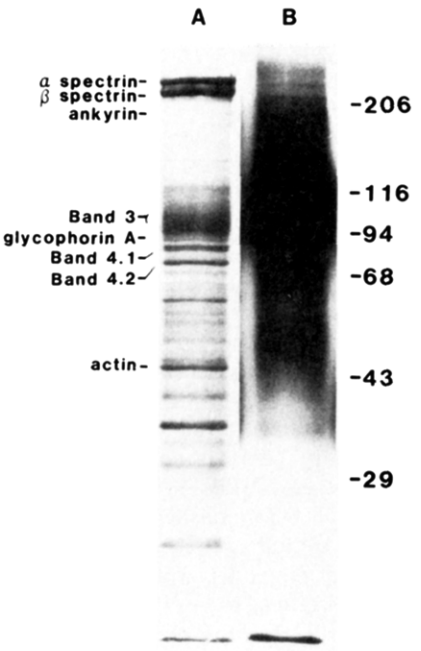

FIGURE 3: Stained gel and autoradiogram of electrophoresed proteins from erythrocytes labeled with ^{125}I-sulfo-SHPP to a high specific activity. A total of 3.5×10^7 washed erythrocytes were labeled with 40 ng of ^{125}I-sulfo-SHPP as described under Materials and Methods. A 0.1-mCi sample of Na^{125}I was used to iodinate 200 ng of sulfo-SHPP. After the iodination of intact cells, ghosts were prepared as described under Materials and Methods. Proteins from these ghosts were run on a 7.5–15% polyacrylamide gel, dried and stained (lane A), and then autoradiographed (lane B).

To further evaluate the degree of membrane penetration by sulfo-SHPP, we analyzed its ability to label specific erythrocyte membrane proteins with known locations on the inner or outer surfaces of the plasma membrane. Erythrocyte ghosts were obtained from intact erythrocytes that were previously labeled with ^{125}I-sulfo-SHPP. The silver/Coomassie Blue double-stained pattern of electrophoresed membrane proteins (Figure 3, lane A) revealed several easily identifiable proteins such as spectrin, ankyrin, glycophorin A, bands 3, 4.1, and 4.2, and actin (Marchesi et al., 1976). Notably, band 3 [a known transmembrane protein exposed to the extracellular environment in erythrocytes (Mueller & Morrison, 1975)] was labeled to a high specific activity in intact erythrocytes with ^{125}I-sulfo-SHPP (lane B) while spectrin, ankyrin, and actin (peripheral membrane proteins on the cytoplasmic face of the plasma membrane) were not labeled in intact erythrocytes. This autoradiogram was purposely overexposed to enhance for the possible detection of cytoplasmic face membrane proteins that were labeled. These proteins are not normally exposed to extracellular ^{125}I-sulfo-SHPP but can be labeled by ^{125}I-sulfo-SHPP when exposed to it as will be shown below. Although it is not readily apparent that ankyrin was not labeled by ^{125}I-sulfo-SHPP in Figure 3, other autoradiograms with less exposure show that anykyrin was not detectably labeled. Because of the proximity of both band 4.1 and band 4.2 to band 3, it could not be determined if they were labeled. These results indicate that ^{125}I-sulfo-SHPP did not permeate the membrane at 4 °C.

Usefulness in the Determination of Membrane Protein Sidedness. Sulfo-SHPP has certain properties that allow its use in the determination of the orientation of proteins in the membrane. First, sulfo-SHPP can be used to selectively label one side of the membrane such as the outer surfce as shown above. Second, there is a finite number of sulfo-SHPP reactive sites on the outer surface of erythrocytes. As shown in Figure

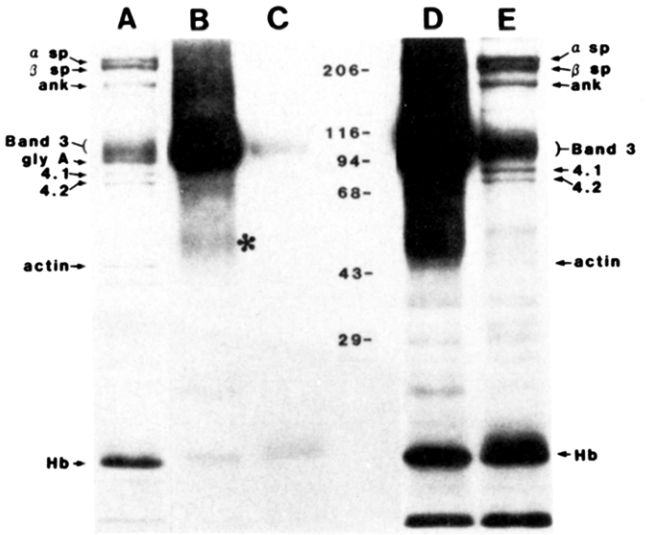

FIGURE 4: Stained gel and autoradiogram of electrophoresed proteins from erythrocytes specifically labeled from either side of the plasma membrane. A total of 3×10^7 washed erythrocytes were labeled with 40 ng of ^{125}I-sulfo-SHPP without (lane B) or with (lane C) pretreatment with 100 μg/mL unlabeled sulfo-SHPP. 1 mCi of $Na^{125}I$ was used to iodinate 200 ng of sulfo-SHPP. Lysed erythrocytes were similarly labeled without (lane D) or with (lane E) pretreatment with 100 μg/mL sulfo-SHPP. Erythrocyte ghosts were prepared and solubilized before electrophoresis on a 7.5–10% polyacrylamide gel. Lane A shows the pattern of stained erythrocyte ghost proteins. α sp, β sp, and, Band 3, gly A, 4.1, 4.2, actin, and Hb show the electrophoretic positions of α-spectrin, β-spectrin, ankyrin, glycophorin A, band 3, band 4.1, band 4.2, actin, and hemoglobin, respectively.

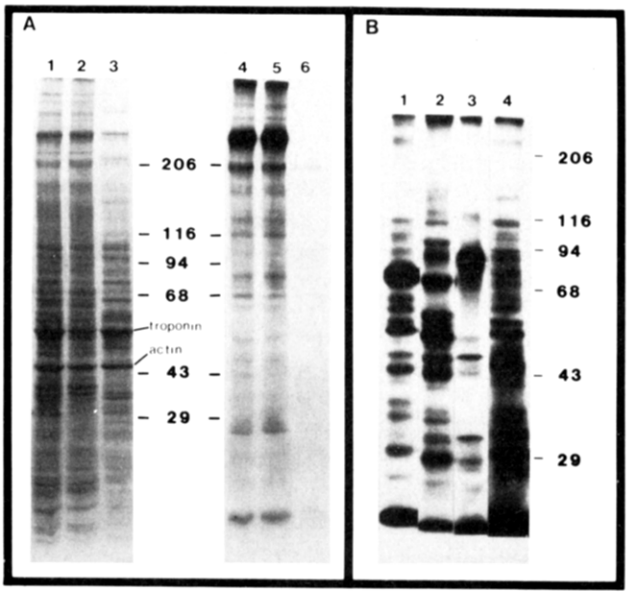

FIGURE 5: Coomassie-stained pattern and autoradiogram of electrophoresed proteins from cultured cells labeled with ^{125}I-sulfo-SHPP. Panel A shows the Coomassie-stained patterns (lanes 1–3) and autoradiogram (lanes 4–6) of proteins from 2×10^6 HF cells labeled with 200 ng of ^{125}I-sulfo-SHPP. 400 ng of sulfo-SHPP was iodinated with 1 mCi of $Na^{125}I$. Approximately 100 μg of protein from the whole cell fraction (lanes 1 and 4), particulate fraction (lanes 2 and 5), and cytoplasmic fraction (lanes 3 and 6) was run on a 7.5% polyacrylamide gel. Panel B shows the autoradiogram of proteins from CHEF cells labeled similarly with ^{125}I-sulfo-SHPP. Proteins from these cells were selectively extracted into four groups as described under Materials and Methods. 1×10^5 cpm of fraction 1 (lane 1), fraction 2 (lane 2), fraction 3 (lane 3), and fraction 4 (lane 4) were electrophoresed on a 7.5% polyacrylamide gel.

2, panel A, the sulfo-SHPP reactive sites on erythrocytes were saturated with ^{125}I-(hydroxyphenyl)propionyl groups at 1 μM ^{125}I-sulfo-SHPP. These amino termini and lysine sites can be reacted with noniodinated sulfo-SHPP to prevent subsequent labeling of them with ^{125}I-sulfo-SHPP. The autoradiogram in Figure 4 compares the effect of reacting the cells with unlabeled sulfo-SHPP (lane C) or not reacting them (lane B) before ^{125}I-sulfo-SHPP treatment. As shown, prelabeling reduced the radioactivity by about 90%. Again, spectrin, ankyrin, and actin, which are peripheral proteins on the cytoplasmic face of the membrane, were not labeled in intact erythrocytes (lane B). At this specific activity, bands 4.1 and 4.2 were also not labeled (lane B).

After blockage of amino groups on the outer surface, erythrocytes were lysed, and the inner surface of the membrane was specifically labeled. Lane D represents proteins labeled on both sides of the membrane; this was produced by labeling lysed cells. Lane E shows proteins from erythrocytes that were reacted with unlabeled sulfo-SHPP, then lysed, and subsequently labeled with ^{125}I-sulfo-SHPP. As shown in Figure 4, lane E, this labeled ankyrin, bands 4.1 and 4.2, actin, and the α and β subunits of spectrin. After blockage of surface amino groups by pretreatment of erthyrocytes with unlabeled sulfo-SHPP, band 3 was readily labeled from its cytoplasmic face after lysing the blocked, intact erythrocytes and treating them with ^{125}I-sulfo-SHPP (compare radioactivity in band 3 of lane E over lane C). Thus, it appears that band 3 can be labeled from either side of the membrane. There was one protein that was labeled from the outer surface of the cell only (asterisk in lane B). The identity of this protein is unknown. Thus, sulfo-SHPP can be used to demonstrate membrane protein sidedness for those proteins with accessible lysines or amino termini.

Labeling of Membrane Proteins on Cultured Human Fibroblasts Using Sulfo-SHPP. The usefulness of ^{125}I-sulfo-SHPP to label cell surface proteins of cultured cells was evaluated by using HF cells. However, analysis of cytoplasmic labeling is more difficult with fibroblasts than with erthyrocytes for two reasons. First, purification of surface membranes from cultured fibroblasts is much more complex than from erythrocytes. Also, harsher conditions must be used to lyse cultured fibroblasts. This results in solubilization of many peripheral membrane proteins. However, an estimate of cytoplasmic labeling can be obtained by observing the extent of labeling of known cytoplasmic proteins such as actin or troponin. Both actin and troponin can be labeled by ^{125}I-sulfo-SHPP after lysis of cells in the absence of 1 mg/mL lysine (data not shown).

To evaluate labeling of membrane proteins, intact HF cells were labeled with ^{125}I-sulfo-SHPP, and particulate and soluble fractions were prepared. Proteins in these fractions were first analyzed by using one-dimensional electrophoresis. Figure 5 (panel A) shows the Coomassie Blue stained gel (lanes 1–3) and the autoradiogram (lanes 4–6) of the gel containing proteins derived from whole cells (lanes 1 and 4), a crude particulate fraction (lanes 2 and 5), and a cytoplasmic fraction (lanes 3 and 6). As shown, the patterns of labeled proteins from whole cells and the crude particulate fraction were identical (lanes 4 and 5). This suggested that membrane proteins were preferentially labeled. Furthermore, actin (43 kDa) and troponin (54 kDa) were easily identified in Coomassie Blue stained gels (lanes 1–3). Importantly, these proteins were not detectably labeled by ^{125}I-sulfo-SHPP in intact cells as shown by autoradiography (lanes 4–6).

To better resolve the labeled membrane proteins, they were fractionated into four groups and then electrophoresed. Labeled membrane proteins can also be run on two-dimensional polyacrylamide gels for analysis (O'Farrell, 1975). However, two-dimensional gels of membrane proteins can be plagued with poor resolution due to the hydrophobicity as well as the

heterogeneous glycosylation of membrane proteins. Alternatively, four groups of labeled membrane proteins were selectively solubilized from whole cells as described under Materials and Methods and electrophoresed on one-dimensional polyacrylamide gels. As shown in panel B of Figure 5, this fractionation produced four distinct groups of labeled proteins with only modest overlap between the groups. Although it was not possible to quantitate the degree of cytoplasmic labeling as it was with erythrocytes, the results of panel A indicated no detectable labeling of cytoplasmic proteins. Thus, the ^{125}I-sulfo-SHPP labeling procedure, along with this fractionation procedure, permitted the visualization of many membrane proteins as reasonably well-resolved bands.

The use of ^{125}I-sulfo-SHPP to study the spacial orientation of membrane proteins in the plasma membrane of tissue culture cells was demonstrated by the labeling and immunoprecipitation of a known transmembrane protein, the EGF receptor (Hunter, 1984). This receptor consists of an exofacial EGF binding domain and an internal tyrosine kinase domain separated by one short transmembranous segment (Hunter, 1984) (shown graphically in Figure 6, panel A). The approach was to visualize the EGF receptor by autoradiography of electrophoretograms after immunoprecipitation from labeled A431 cells with a monoclonal antibody. In some cases, cells were pretreated with unlabeled sulfo-SHPP before labeling to block exposed amino termini, or cells were pretreated and then lysed before labeling. After labeling of A431 cells with ^{125}I-sulfo-SHPP, autoradiograms of immunoprecipitated EGF receptor revealed a labeled band at 170 K as shown in Figure 6, lane 2. The efficiency of blockage of cell surface amino groups was demonstrated by pretreatment of A431 cells with unlabeled sulfo-SHPP followed by labeling with ^{125}I-sulfo-SHPP and subsequent immunoprecipitation of the EGF receptor. As shown in Figure 6, lane 5, the EGF receptor was labeled to a much lesser extent if free amino groups were blocked before labeling with ^{125}I-sulfo-SHPP. An equivalent amount of the EGF receptor was precipitated in each case since intensities of Coomassie Blue stained bands were the same in all lanes (data not shown). Specific labeling of the cytoplasmic domain of the EGF receptor was accomplished by blockage of cell surface amino groups on intact cells. Proteins on the inner surface of the membrane were then labeled after hypotonic lysis of the cells. Even though the exofacial amino groups of the EGF receptor were largely blocked and unable to be labeled in intact cells as shown in lane 5 of Figure 6, the EGF receptor was labeled on the cytoplasmic domain after lysis of the cells. This was shown by the increase in radioactivity incorporated into the 170 K receptor labeled after lysis (lane 8) compared to before lysis (lane 5). This demonstrates the utility of sulfo-SHPP in the study of the EGF receptor as a transmembrane protein, since it can be labeled from both the extracellular and cytoplasmic sides of the membrane.

An alternate possibility for the increased labeling of the EGF receptor in lane 8 over lane 5 was that lysis of the A431 cells caused an increase in the number of EGF receptors at the cell surface that were not prelabeled by sulfo-SHPP. The possibility was excluded by cleaving the EGF receptor into a 115 K exofacial EGF binding domain and an internal 30 K or 40 K tyrosine kinase domain by treatment with trypsin (Hunter, 1984) (shown graphically in Figure 6, panel A). Cells were treated and labeled as described above, except after immunoprecipitation the EGF receptor was treated with trypsin. The 115 K exofacial tryptic fragment was radiolabeled by ^{125}I-sulfo-SHPP on intact cells (lane 3 of Figure 6). Pre-

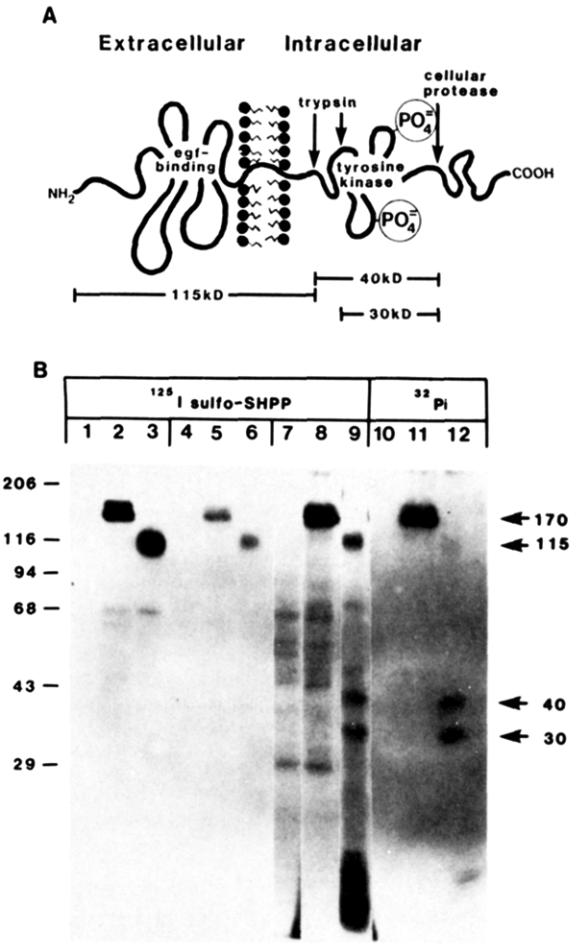

FIGURE 6: Labeling of the EGF receptor from the extracellular and cytoplasmic faces of the plasma membrane. Panel A contains a schematic representation of the EGF receptor. Shown are the extracellular EGF binding domain, the intracellular tyrosine kinase domain, and approximate positions of the trypsin cleavage and some autophosphorylation sites (Hunter, 1984). Panel B shows an autoradiogram of immunoprecipitated EGF receptor from radiolabeled A431 cells. Lanes 1–9 are derived from A431 cells labeled with ^{125}I-sulfo-SHPP. Confluent A431 cells in a 100-mm tissue culture dish were iodinated with 0.333 μg of ^{125}I-sulfo-SHPP in 5 mL of D-PBS. 2 mCi of Na^{125}I was used to iodinate 1 μg of sulfo-SHPP (lanes 1–3). A second culture of A431 cells was similarly labeled after pretreatment with 2 mg/mL unlabeled sulfo-SHPP (lanes 4–6). A third culture was similarly labeled after pretreatment with 2 mg/mL unlabeled sulfo-SHPP and then hypotonic lysis (lanes 7–9). A fourth culture of confluent A431 cells was labeled by incubation of the cells with 12 μCi/mL [^{32}P]P_i for 1 h, and then 1 ng/mL EGF was added for 10 min at 37 °C. Membrane proteins were solubilized as described under Materials and Methods. The labeled proteins from each culture were separated into three equal aliquots. The first aliquot from each sample was a control which received no monoclonal antibody against the EGF receptor (lanes 1, 4, 7, and 10). The second aliquot was immunoprecipitated with the antireceptor antibody (lanes 2, 5, 8, and 11). The third aliquot was immunoprecipitated with the antireceptor antibody, and the immunoprecipitate was treated with 2.5 μg/mL TPCK-treated trypsin at 22 °C for 30 min before electrophoresis on a 7.5% polyacrylamide gel (lanes 3, 6, 9, and 12).

treating intact cells with unlabeled sulfo-SHPP on intact cells (lane 3 of Figure 6). Pretreating intact cells with unlabeled sulfo-SHPP reduced subsequent labeling of this exofacial 115 K fragment by ^{125}I-sulfo-SHPP (lanes 3 and 6), suggesting that it is indeed on the extracellular side of the membrane. The 30 K or 40 K internal domain was not labeled on intact cells as shown by their absence in lanes 3 and 6. However, if cells were pretreated with unlabeled sulfo-SHPP, lysed, and then labeled with ^{125}I-sulfo-SHPP, the 30 K and 40 K fragments resulting from trypsin treatment were specifically ra-

diolabeled as shown in lane 9, showing that the EGF receptor was indeed labeled on the cytoplasmic side of the membrane. Confirmation that the 30 K or 40 K fragment was the cytoplasmic tyrosine kinase domain was demonstrated by autophosphorylation of the EGF receptor in cells preincubated with [^{32}P]P_i. In the presence of EGF, the EGF receptor phorphorylates itself on the cytoplasmic domain (Hunter, 1984). Indeed, as shown in Figure 6, [^{32}P]P_i was incorporated in both the intact receptor (lane 11) as well as the 30 K or 40 K tryptic fragment (lane 12). Therefore, these techniques can be used to study the spatial orientation of proteins in the plasma membrane of cultured cells as well as erythrocytes.

Discussion

^{125}I-Sulfo-SHPP can be used for selective labeling of membrane proteins to a high specific activity. This procedure was shown to be useful for both erythrocytes and cultured fibroblast-like cells. As was demonstrated on erythrocytes, the specific labeling of membrane proteins by ^{125}I-sulfo-SHPP has certain advantages over the use of lactoperoxidase and [^{125}I]iodide. First, the labeling of cells with ^{125}I-sulfo-SHPP occurs efficiently at 4 °C. At this temperature, many cellular processes are slowed or halted. For example, endocytosis of membranes or the insertion of new membrane proteins into the plasma membrane does not occur under 15 °C (Steinman et al., 1974). Iodination at 4 °C allows for the study of membrane proteins "frozen" at a particular physiological state without the interference of membrane protein turnover. With lactoperoxidase, iodination occurs more slowly at 4 °C than at 22 °C; this results in labeling of membrane proteins to a lower specific activity (Hubbard & Cohn, 1975). Another advantage of sulfo-SHPP is that it can label membrane proteins to a very high specific activity with little labeling of cytoplasmic proteins when compared to the lactoperoxidase and [^{125}I]iodide technique (Figure 2). I_2 has been shown to permeablize the membrane (Markwell & Fox, 1978), thus allowing access to cytoplasmic proteins by labeling reagents. When iodination proceeds too rapidly, as with high concentrations of chloroglycoluril, a high concentration of $^{125}I_2$ is formed (Markwell & Fox, 1978). Although not presented here, we found that high concentrations of [^{125}I]iodide during lactoperoxidase iodination cause permeablization of the membrane of tissue culture cells. However, large amounts of sulfo-SHPP can be added without affecting the integrity of the membrane. This was shown by the lack of hemoglobin labeling at high ^{125}I-sulfo-SHPP concentrations and also by the fact that over 2 mg/mL sulfo-SHPP can be added to erythrocytes without evidence of hemolysis (data not shown).

An additional advantage of sulfo-SHPP-mediated iodination is the ability to selectively label proteins on the inner surface of the plasma membrane. The finite number of reactive amino groups on the outer surface of the plasma membrane can be blocked by preincubation of intact cells with unlabeled sulfo-SHPP. After this blockage, labeling of exofacial proteins by ^{125}I-sulfo-SHPP was markedly reduced. If the cells with blocked amino groups are lysed, proteins on the inner surface can be iodinated specifically (Figure 4). Other investigators have iodinated proteins on the cytoplasmic face of the plasma membrane with lactoperoxidase but only after production of purified inside-out vesicles (Steck et al., 1970). The procedure presented here offers a simple alternative. As shown in this study, proteins known to exist exclusively at the cytoplasmic face of erythrocyte membranes were labeled after blockage of surface amino groups. Transmembrane proteins such as band 3 can be labeled from either side of the plasma membrane (Figure 5, lanes B and E).

One difficulty in studies on membrane proteins is the inability to effectively resolve the complex mixtures of proteins present in the plasma membrane. Many methods have been proposed to separate membrane proteins electrophoretically, such as on two-dimensional polyacrylamide gels (O'Farrell, 1975) or as shown here and elsewhere by electrophoresis after fractionation into four groups by selective extraction. This latter technique has been shown to resolve many components of the membrane visualized by silver staining of electrophoretograms (Thompson & Cunningham, 1987). However, detection of membrane proteins by staining polyacrylamide gels is highly dependent on the purity of the plasma membrane which frequently has subcellular organelles attached to it. By labeling membrane proteins with the highly selective ^{125}I-sulfo-SHPP procedure, membrane proteins can be electrophoresed and detected by autoradiography without the need for prior purification of the plasma membrane. Combined with the procedure to selectively fractionate membrane proteins into four groups and resolve them on one-dimensional gels, it was possible to effectively resolve many membrane proteins of cultured fibroblasts.

Analysis of the orientation of proteins in the membranes of tissue culture cells can also be conducted by using ^{125}I-sulfo-SHPP. However, after iodination of lysed tissue culture cells, proteins on the cytoplasmic surface of the plasma membrane as well as other cytoplasmic proteins are labeled. After electrophoresis, cytoplasmic proteins cannot be distinguished from membrane proteins without prior purification of membranes. As presented here (Figure 6), immunoprecipitation of specific proteins like the EGF receptor can circumvent this problem. If a protein is specifically labeled from either side of the plasma membrane, it is a transmembrane protein. The EGF receptor, a known transmembrane protein, was specifically labeled from both sides of the plasma membrane (Figure 6). The use of proteolysis can corroborate the determination of a protein's position in the membrane. Limited proteolysis of the EGF receptor with trypsin cleaves the transmembrane protein into exofacial and cytoplasmic domains, which were specifically labeled on the appropriate side of the membrane (Figure 6).

We are presently optimizing the conditions for other uses of sulfo-SHPP. Preliminary studies show that sulfo-SHPP can be used as a sensitive method to detect proteolytic cleavage products at cell surfaces. Blockage of existing amino groups on cells prevents exofacial membrane proteins from being labeled. "New" amino termini are produced by treatment of the cells with a proteolytic enzyme. After such treatment, proteolytic products at the cell surface can then be labeled, since they have the only available amino groups. The use of sulfo-SHPP for membrane protein turnover studies as well as the ability to identify receptors after protection of a receptor's exofacial amino groups by its ligand is presently being investigated. It should be pointed out that sulfo-SHPP reacts with amino groups, thereby changing the charge of the protein, and this may affect that protein's conformation or activity. This precaution should be considered in the above applications of this reagent.

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