Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lovell, S. J., & Harrington, W. F. (1981) *J. Mol. Biol. 149*, 659–674.
Matsubara, I., Yagi, N., Miura, H., Ozeki, M., & Izumi, T. (1984) *Nature (London) 312*, 471.
Mornet, D., Pantel, P., Audemard, E., & Kassab, R. (1979) *Biochem. Biophys. Res. Commun. 89*, 925–932.
Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306.
Reisler, E. (1982) *Methods Enzymol. 85*, 84–93.
Rome, E. (1967) *J. Mol. Biol. 27*, 591–602.
Sekine, T., & Kielley, W. W. (1964) *Biochim. Biophys. Acta 81*, 336–341.
Sutoh, K., & Harrington, W. F. (1977) *Biochemistry 16*, 2441–2449.
Yamamoto, K., & Sekine, T. (1979) *J. Biochem. (Tokyo) 86*, 1855–1862.
Yanagida, T. (1981) *J. Mol. Biol. 146*, 539–560.

Detection of Nearest Neighbors to Specific Fluorescently Tagged Ligands in Rod Outer Segment and Lymphocyte Plasma Membranes by Photosensitization of 5-Iodonaphthyl 1-Azide†

Yosef Raviv,‡ Tuvia Bercovici,§ Carlos Gitler,§ and Yoram Salomon*,‡

Departments of Hormone Research and Membrane Research, The Weizmann Institute of Science, Rehovot 76100, Israel

Received March 22, 1988; Revised Manuscript Received September 8, 1988

ABSTRACT: Lima bean agglutinin–fluorescein 5-isothiocyanate conjugate (FluNCS–lima bean lectin) interacts with specific receptor molecules on membranes both from the rod outer segment (ROS) of the frog retina and from S_{49} mouse lymphoma cells. When [^{125}I]-5-iodonaphthyl 1-azide (^{125}I-INA), which freely and randomly partitions into the lipid bilayer, is added to membranes and the suspension is irradiated at 480 nm, the FluNCS-conjugated lectin photosensitizes the [^{125}I]INA but only at discrete sites. This results in the selective labeling of specific proteins: an 88-kDa protein on ROS membranes and a 56-kDa protein on S_{49} plasma membranes. Labeling is dependent upon the interaction of the FluNCS–lectin with glycosylated receptor sites, since *N*-acetylgalactosamine, but not methyl α-mannoside, blocked labeling of the 56-kDa protein on S_{49} membranes. In contrast, a random labeling pattern of membrane proteins was observed upon irradiation at 480 nm using other fluorescein conjugates, such as FluNCS–bovine serum albumin (FluNCS–BSA) or FluNCS–soybean trypsin inhibitor (FluNCS–STI), which interact with cell membranes in a nonselective manner, or with *N*-(fluorescein-5-thiocarbamoyl)-*n*-undecyclamine (FluNCS-NHC_{11}), which is freely miscible in the membrane lipid. Random labeling was also obtained by direct photoexcitation of [^{125}I]INA at 314 nm, with no distinct labeling of the 88- and 56-kDa proteins in the respective membranes. These results suggest that protein ligands can be used to guide sensitizers to discrete receptor sites and lead to their selective labeling by photosensitized activation of [^{125}I]INA [Raviv, Y., Salomon, Y., Gitler, C., & Bercovici, T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6103–6107]. Site-directed labeling is obtained by an amplification process that locally and time-dependently intensifies the radioactive signal, thus revealing minor membranal components that could not otherwise be visualized by random labeling. This approach provides a method that offers new possibilities for application in different fields of chemical and biological research.

Elucidation of the dynamic pattern of interactions among molecules in highly complex cell membranes, or other biological structures, is an extremely difficult task. Progress in the understanding of the processes that underly these interactions requires methods of ever higher resolving power in terms of both time and space. For this purpose, techniques that permit the analysis of spatial proximity of macromolecules and identification of the nearest-neighbor components have been developed. These include chemical and photochemical cross-linking, affinity or photoaffinity labeling, and topological labeling. These techniques use NaDodSO$_4$–PAGE[1] as a tool for the resolution and isolation of the proteins which have been radioactively labeled or otherwise tagged. In chemical cross-linking, bifunctional reagents are reacted with the membrane and the cross-linked products formed are analyzed by NaDodSO$_4$–PAGE. Cleavable cross-linking reagents enable the regeneration of the cross-linked components. This technique has been very successfully applied in biological

† This paper has been submitted by Y.R. in partial fulfillment of the Ph.D. Thesis, Feinberg Graduate School, The Weizmann Institute of Science. Y.S. is the Charles W. and Tillie Lubin Professor of Hormone Research. C.G. is the E. Stanley Enlund Professor of Membrane Biology.

* To whom correspondence should be addressed.
‡ Department of Hormone Research.
§ Department of Membrane Research.

[1] Abbreviations: αMM, methyl α-mannoside; BSA, bovine serum albumin; Con A, concanavalin A; FluNCS, fluorescein 5-isothiocyanate; FluNCS-NHC_{11}, *N*-(fluorescein-5-thiocarbamoyl)-*n*-undecylamine; INA, 5-iodonaphthyl 1-azide; NaDodSO$_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NAGA, *N*-acetylgalactosamine; PSL, photosensitized labeling; PBS, phosphate-buffered saline; ROS, rod outer segment.

0006-2960/89/0428-1313$01.50/0

research and has provided a great deal of information [for review see Peters and Richards (1977), Ji (1979), Ji and Middaugh (1980), Ross et al. (1982), and Middaugh et al. (1983)]. The limitation of these techniques is that formation of cross-links depends on rather stable association of the participating molecules and that the process generates a host of cross-linked products that are sometimes difficult to analyze and identify.

Affinity and photoaffinity labeling techniques provide a means by which radiolabeled ligands, derivatized with a chemical or photochemical reactive group, are employed to identify a component in a complex biological system to which it binds. Photoaffinity labeling proved very efficient and was applied successfully on numerous occasions especially in study of enzyme–substrate and hormone–receptor interaction [for review see Chowdry and Westheimer (1979), Czarnecki et al. (1979), Tometsko and Richards (1980), Guillory and Jeng (1977), Bayley and Knowles (1977), Guillory (1979), Hazum (1983), Fedan et al. (1984), and Bayley and Staros (1984)]. The difficulties that often arise in photoaffinity labeling are nonspecific labeling and poor efficiency of the labeling process and that a rather stable complex is performed. Topological labeling was applied to obtain information about the accessibility of a certain molecule to a specific cellular compartment (Gitler & Klip, 1974; Klip & Gitler, 1976; Brunner, 1981; Bayley, 1983). 5-Iodonaphthyl 1-azide (INA), along with other lipophilic photoreactive compounds, was successfully used for labeling proteins/enzymes from within the hydrocarbon core of biological membranes (Klip & Gitler, 1974; Klip et al., 1976; Bercovici & Gitler, 1978; Sidman et al., 1980; Brunner & Semenza, 1981; Jørgensen et al., 1982; Raviv et al., 1984; Davison & Findlay, 1986). In order to extend the application of INA and related aryl azides, we recently introduced a new approach by which only a discrete fraction of the azide molecules can be selectively activated by photosensitization (Raviv et al., 1987). By use of photosensitizers, such as fluorescein isothiocyanate or the intrinsic retinal of rhodopsin, it was demonstrated that selective labeling of chromophore-bearing proteins could be obtained by photosensitized labeling (PSL).

In this study, we demonstrate that the PSL technique can be used for targeting [^{125}I]INA labeling to specific receptor sites using ligand–sensitizer conjugates. It is shown that, with this approach, membrane proteins could be specifically labeled and identified with high efficiency, without the cross-linking of the participating components. It is suggested that this application of PSL may prove efficient for identification and labeling of nearest-neighbor components even upon transient encounters and may provide advantages not present in other techniques for the study of the specific interactions of molecules, cells, or other particles in the most general sense.

Experimental Procedures

Materials

FluNCS–BSA, FluNCS–lima bean agglutinin, FluNCS, soybean trypsin inhibitor (STI), *N*-acetylgalactosamine, and methyl α-mannoside were from Sigma Chemical Co. 1-Amino-5-azidonaphthalene was from Fluka. Carrier-free Na^{125}I was from New England Nuclear. All other reagents were of analytical grade.

Methods

Synthesis of INA and General Conditions for Its Use. [^{125}I]INA (2–10 Ci/mmol: 1 Ci = 37 GBq) was synthesized from 1-amino-5-azidonaphthalene as described earlier (Bercovici & Gitler, 1978). All operations in which INA was used, including $NaDodSO_4$–PAGE, were performed under subdued light ($\lambda > 600$ nm). INA and sensitizers were added as ethanol or dimethyl sulfoxide solutions; final solvent concentrations was <1%.

Treatment of Membranes with [^{125}I]INA. S_{49} lymphoma cell membranes or membranes from rod outer segments (ROS) of frog retina were suspended in phosphate-buffered saline (PBS) or 25 mM Tris-HCl buffer, pH 7.5, as indicated below. Different amounts of membrane protein, as described for each experiment, were mixed in 6 × 50 mm test tubes with (2–3) × 10^6 cpm of [^{125}I]INA (0.7–1 Ci/mmol), and the mixture was incubated on ice for 5 min. As specified for each experiment, the membranes were subjected to irradiation at 314 or 480 nm as described below.

Irradiation Conditions. Samples were irradiated at 314 nm for direct excitation of INA or at 480 nm for photosensitization of the compound in the presence of fluorescein derivatives. The irradiation was carried out by using the identical conditions described previously (Raviv et al., 1987). Irradiation time at 314 nm was 2 min and at 480 nm was 15 min.

Preparation of FluNCS–STI. STI (0.5 μmol) in 1 mL of 0.1 M phosphate buffer, pH 9.0, was mixed with 10 equiv of FluNCS. The mixture was incubated for 1 h at room temperature and subsequently separated from unreacted FluNCS by gel filtration using Sephadex G-25.

Synthesis of N-(Fluorescein-5-thiocarbamoyl)-n-undecylamine (FluNCS-NHC$_{11}$). Material was prepared as previously described (Raviv et al., 1987).

Preparation of Plasma Membranes. Mouse S_{49} lymphoma plasma membranes were prepared as described before (Ross et al., 1977). Rod outer segment (ROS) membranes were prepared from frog (*Rana ridibunda*) retinas, as previously described (McDowell & Kuhn, 1977).

Photosensitization of [^{125}I]INA in the Membrane Using FluNCS-Conjugated Proteins. All operations were carried out in a dark room under subdued red light. S_{49} lymphoma cell plasma membranes were suspended in PBS to a concentration of 1 mg/mL. Membrane suspension (70 μg of protein) was taken for each experimental group. The membranes in each group were treated with [^{125}I]INA as described above. Subsequently, FluNCS–BSA or FluNCS–STI were added to a final concentration of 0.5 mg/mL. To control groups without FluNCS–conjugate, a respective volume of PBS was added to a final volume of 150 μL. The membranes were then subjected to irradiation at either 314 or 480 nm, as described above. Membranes of all groups were then washed by centrifugation (10 min at 100000*g*) using a Beckman airfuge and subjected to $NaDodSO_4$–PAGE (Laemmli, 1970), followed by autoradiography using Kodak XAR-5 diagnostic film. In addition, either FluNCS–STI or FluNCS–BSA was mixed with [^{125}I]INA, irradiated at 480 nm, and subjected to $NaDodSO_4$–PAGE as controls for self-PSL. The intensity of radioactive labeling of membrane proteins was dependent on the degree of photoactivation of [^{125}I]INA, which was higher following direct irradiation (314 nm) than photosensitization (480 nm) of [^{125}I]INA. Consequently, different exposure times of the film at −130 °C were required, as specified.

FluNCS–Lima Bean Lectin Induced [^{125}I]INA Labeling of Membrane Proteins. ROS or S_{49} lymphoma cell membranes were suspended in 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$. Each membrane suspension was divided into four experimental groups (50 μg of ROS membrane protein or 80 μg of S_{49} membrane protein per sample) in a final volume of 150 μL. FluNCS–lima bean

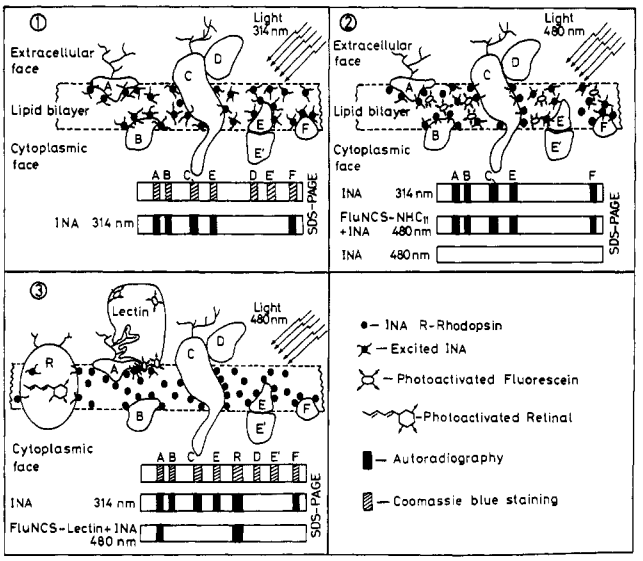

FIGURE 1: Photosensitized labeling of proteins with [^{125}I]INA: a scheme. Part 1: Labeling of membrane proteins by direct photoactivation of [^{125}I]INA at 314 nm. Part 2: Labeling of membrane proteins by photosensitization of [^{125}I]INA using a randomly distributed photosensitizer, FluNCS-NHC$_{11}$. Part 3: FluNCS–ligand-induced photosensitized labeling of specific membrane proteins and photosensitized labeling of native chromophore (retinal) containing rhodopsin in ROS membranes.

lectin (75 μg) or FluNCS-NHC$_{11}$ (1 nmol) was added to the membranes and incubated for 1 h at room temperature. Subsequently, membranes were treated with [^{125}I]INA and irradiated at 480 nm or at 314 nm as described above. The membranes were then washed once and subjected to NaDodSO$_4$–PAGE followed by autoradiography, as described above.

Results

Photosensitized Labeling of Proteins and Their Neighbors: A Scheme. A scheme presenting the principle of the approach for labeling of proteins and their nearest neighbors in biological membranes by PSL is presented in Figure 1. In one application of the process (Figure 1, part 1), a photolabile radioactive compound like [^{125}I]INA (lipid/water partition coefficient $\sim 10^5$) is allowed to partition into the membrane in the dark. Upon irradiation at 314 nm INA undergoes photolysis to yield nitrenes and other reactive derivatives which react with, and covalently label, adjacent membrane proteins. As shown previously (Bercovici & Gitler, 1978; Klip et al., 1976; Sidman et al., 1980; Raviv et al., 1987), only those proteins that contain lipid-embedded domains (A–C, E, and F) will become labeled while other proteins (D and E′) will not (on the schematic presentation of NaDodSO$_4$–PAGE compare Coomassie brilliant blue staining with autoradiographic patterns of the appropriate lanes). In another application of the process (Figure 1, part 2) a suitable lipid-soluble photosensitizer chromophore like FluNCS-NHC$_{11}$ is permitted to partition together with INA into the lipid phase of the membrane in the dark. Upon irradiation of the membrane at 480 nm (a wavelength absorbed only by the photosensitizer), [^{125}I]INA is indirectly photoactivated by sensitization, leading to a pattern of radioactive protein labeling identical with that obtained by direct photoactivation of [^{125}I]INA at 314 nm (see also Figure 1, part 1). In contrast, irradiation at 480 nm of membranes containing [^{125}I]INA in the absence of photosensitizer will not reveal any radioactive labeled proteins (blank lane, NaDodSO$_4$–PAGE). In a third application of this process (Figure 1, part 3), a natural photosensitizer chromophore (retinal) covalently linked to rhodopsin (R) in ROS membranes will, upon photosensitization, restrict [^{125}I]INA labeling only to itself, as described by us previously (Raviv et al., 1987). Likewise, tagging of a lectin with photosensitizer (FluNCS) will target [^{125}I]INA labeling to a specific binding site identified by the lectin (protein A; Figure 1, part 3). In this case, autoradiography of the membrane proteins on NaDodSO$_4$–PAGE will reveal that only discrete proteins linked in proximity to the photosensitizer will become radioactively labeled. Since the photoactivated derivatives of [^{125}I]INA bind covalently to proteins, it is expected that continuous irradiation and steady diffusion of nonactivated [^{125}I]INA molecules into the sink formed around the chromophore will lead to a gradual drainage of INA from the bilayer. Consequently, the photosensitized [^{125}I]INA will be selectively introduced into protein domains adjacent to the photosensitizer in a process that will progressively intensify their radioactive labeling. Not included on the schematic presentations of the NaDodSO$_4$–PAGE lanes is the self-labeling of the chromophore–ligand itself which is also expected to become labeled in this process (Raviv et al., 1987).

The following experiments were designed to test the feasibility of the principles described in Figure 1 and to examine this approach for its efficiency to induce selective photosensitized [^{125}I]INA labeling of nearest-neighbor membrane proteins using FluNCS–protein conjugates as photosensitizers.

FluNCS-Conjugated Proteins Induce Photosensitized [^{125}I]INA Labeling of Plasma Membrane Proteins. We first examined whether labeling of membrane proteins by photosensitization of [^{125}I]INA can be achieved with sensitizers that are conjugated to exogenously added soluble proteins. For this purpose two different FluNCS-conjugated proteins, FluNCS–BSA and FluNCS–STI, were each mixed into a suspension of S$_{49}$ lymphoma cell plasma membranes, which was previously allowed to interact with [^{125}I]INA. Subsequently, the suspensions were irradiated at 480 nm (a wavelength absorbed by FluNCS) and the radioactive labeling pattern of the membrane proteins was examined. The results reveal that FluNCS covalently linked to either BSA (Figure 2, lane 3) or STI (Figure 2, lane 4) allows for the labeling of membrane proteins by photosensitization. Furthermore, the labeling pattern was very similar to that obtained by direct excitation of [^{125}I]INA at 314 nm (Figure 2, lane 1). However, no protein labeling was obtained upon irradiation at 480 nm in the absence of sensitizer (Figure 2, lane 2). Thus, these chromophore–protein conjugates absorb to membranes to an extent that permits photosensitization of INA in the membrane and seem to behave essentially like other lipophilic photosensitizers which randomly distribute in the lipid phase of the membrane (Figures 1, part 2, and 3, lane 3) (Raviv et al., 1987). In agreement with previous observations (Raviv et al., 1987), the FluNCS–protein conjugates themselves also became labeled due to photosensitization of tightly adsorbed INA (Figure 2, lanes 5 and 6). Consequently, traces of these proteins which were not removed by washing can still be detected on the autoradiogram of the membranes in Figure 2, lanes 3 and 4.

Selective Photosensitized [^{125}I]INA Labeling As Induced by FluNCS–Lima Bean Lectin. It was now of interest to test whether fluorescein conjugated to a ligand protein, which is known to interact with specific binding sites on the membrane, will confer selective labeling to its putative receptor sites, in agreement with the principle proposed in Figure 1, part 3. In this experiment FluNCS–lima bean lectin was allowed to bind to membrane preparations from two different sources: S$_{49}$

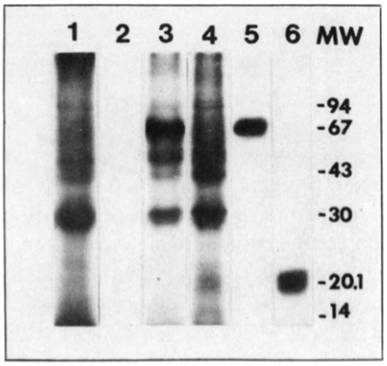

FIGURE 2: Photosensitization of [^{125}I]INA in the membrane using FluNCS-conjugated proteins. A suspension of S_{49} lymphoma cell membranes was divided into four experimental groups marked 1–4, and each group was subjected to further treatment as specified below. The membranes were then washed and subjected to $NaDodSO_4$–PAGE (10%), followed by autoradiography as described under Methods. Each lane represents a different experimental group. The Coomassie blue staining patterns of the proteins of all experimental groups were identical. Thus, only the radioactive labeling pattern as revealed by autoradiography is presented. The radioactive lanes represent different exposure times of the film: 2 h (lanes 1, 5, and 6), 5 h (lane 4), and 14 h (lanes 2 and 3). Lane 1: S_{49} lymphoma cell membranes treated with [^{125}I]INA and irradiated at 314 nm for direct photoactivation of the radiolabeled compound. This group permits examination of the general [^{125}I]INA labeling pattern of membrane proteins. Lane 2: Membranes treated as described in lane 1 except that irradiation was carried out at 480 nm. This control group was used for evaluation of backgroun labeling under conditions that do not induce direct photoactivation of [^{125}I]INA. Lane 3: FluNCS–BSA was added to the membranes after they were treated with [^{125}I]INA as described for lane 1 and were subsequently irradiated at 480 nm as in lane 2. This group is used for evaluation of the labeling pattern obtained by photosensitization of [^{125}I]INA in the membrane using the FluNCS–BSA conjugate as the photosensitizer. Lane 4: As in lane 3 except that FluNCS–STI was used as the photosensitizer. Lane 5: The FluNCS–BSA preparation (5 μg) was mixed with [^{125}I]INA in PBS and irradiated at 480 nm. This lane is used as a marker for FluNCS–BSA migration and as a control for self-PSL of the chromophore–lectin conjugate. Lane 6: The same as lane 5 except that 5 μg of FluNCS–STI was used as a marker.

mouse lymphoma cell membranes and rod outer segment (ROS) membranes from the frog retina. Subsequently, the membranes were washed, treated with [^{125}I]INA, and irradiated at 480 nm for photoactivation of the bound FluNCS–lectin. As can be seen in Figure 3, the FluNCS–lectin conjugate induced labeling of discrete proteins, one each on the respective membrane preparation. An 88-kDa protein on ROS membranes (Figure 3, ROS, lane 4 at arrow) and a 56-kDa protein in lymphoma cell membranes (Figure 3, S_{49}, lane 4 at arrow) were labeled. These two proteins are almost undetectable on the autoradiograms on the respective membranes as labeled by direct photoactivation of [^{125}I]INA at 314 nm (Figure 3, ROS and S_{49}, lanes 1), or in membranes labeled by photosensitization of [^{125}I]INA using randomly distributed FluNCS-NHC_{11} as the sensitizer (Figure 3, ROS and S_{49}, lanes 3). FluNCS-NHC_{11} is a lipophilic probe that freely partitions into the membrane and thus becomes evenly distributed throughout the lipid bilayer (Raviv et al., 1987). In control membranes, which were treated with [^{125}I]INA and irradiated at 480 nm in the absence of photosensitizer, no radioactive labeling of proteins was observed (Figure 3, S_{49}, lane 2; see also Figure 1, part 2). However, ROS membranes clearly present an exception in this regard because, as we have shown before (Raviv et al., 1987), rhodopsin—the major chromophore-bearing protein—induces, upon irradiation at 380 or 480 nm, self-labeling by the photosensitization of [^{125}I]INA (Figure 3, ROS, lane 2, and Figure 1, part 3, protein

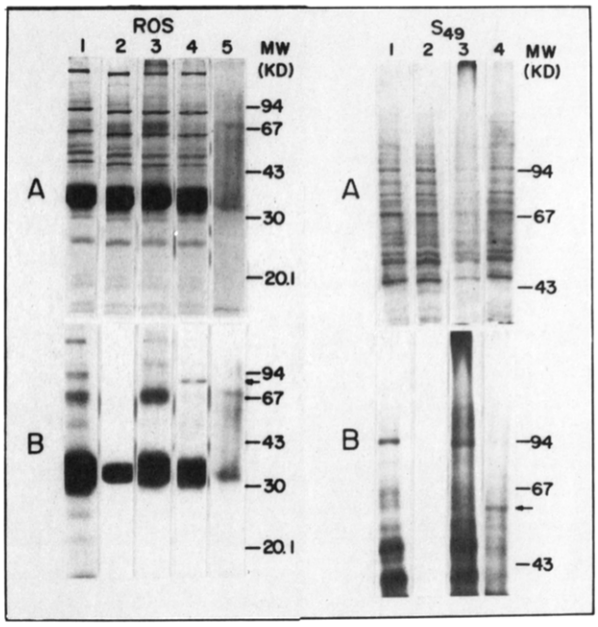

FIGURE 3: Photosensitized [^{125}I]INA labeling of specific membrane proteins as induced by FluNCS–lima bean lectin. Purified ROS membranes and plasma membranes from S_{49} mouse lymphoma cells were divided into four experimental groups each and treated as described below. At the end of the experiment the membranes were subjected to $NaDodSO_4$–PAGE followed by autoradiography. ROS membrane proteins were separated on a 10% gel and S_{49} cell membrane proteins on a 6% gel. Each lane on the gel represents a different experimental group. Parts A show the Coomassie brilliant blue staining pattern of the membranes. Parts B show the autoradiography of the gels in parts A. All other details were as described under Methods. Lane 1: Membranes treated with [^{125}I]INA and irradiated at 314 nm for direct photoactivation of the radioactive compound. Lane 2: [^{125}I]INA-treated membranes are irradiated at 480 nm, a wavelength at which INA does not absorb and is thus not excited. Lane 3: FluNCS-NHC_{11} (1 nmol) was added together with [^{125}I]INA, and the membranes were subsequently irradiated at 480 nm. Lane 4: FluNCS–lima bean lectin was allowed to bind to both types of membranes in the presence of [^{125}I]INA, and the membranes were subsequently irradiated at 480 nm. Lane 5: [^{125}I]INA labeling pattern of the commercial FluNCS–lima bean lectin preparation used in this experiment (control).

C). By comparing the intensity of radioactive labeling of the 88-kDa protein in ROS membranes and 56-kDa protein in S_{49} membranes (Figure 3, lanes 3) with the respective counterparts labeled by direct excitation of [^{125}I]INA (Figure 3, lanes 1), the principle of local amplification of the radioactive signal at the site of photosensitization is clearly demonstrated (cf. Figure 1, part 3).

Inhibition by N-Acetylgalactosamine of FluNCS–Lima Bean Lectin Induced Photosensitization. We further examined whether the selective labeling of these proteins is indeed dependent on the interaction of the lectin with specific glycosylated binding sites. For this purpose, displacement experiments were carried out with NAGA, which binds selectively to lima bean lectin, and thus specifically blocks its interaction with putative receptor sites. In an experiment conducted as described in Figure 3, S_{49} lymphoma cell membranes were subjected to [^{125}I]INA labeling by photosensitization using FluNCS–lima bean lectin, and results were quantitatively analyzed by densitometric scanning of the autoradiograms (Figure 4, part II). Upon inclusion of NAGA, the labeling of a single peak b was eliminated (Figure 4, part III). This peak is identified as the 56-kDa protein (Figure 3, S_{49}, lane 4). Upon inclusion of αMM, which is not recognized by lima bean lectin, labeling of peak b was unimpaired (Figure 4, part IV). The pattern of labeling obtained by direct photoactivation

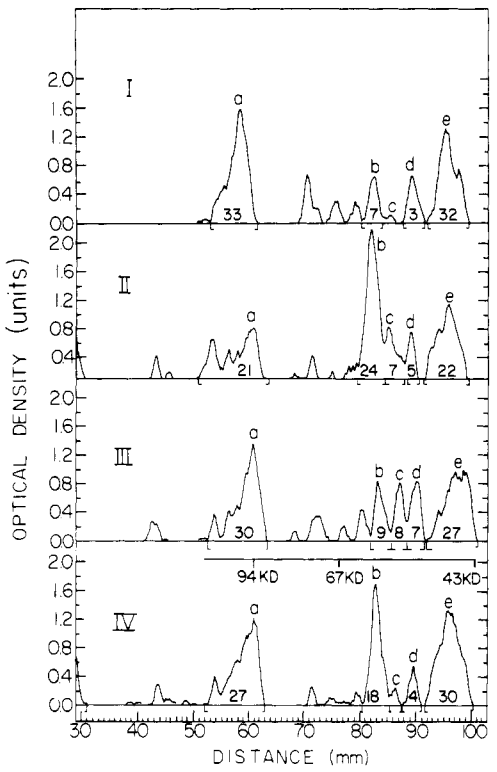

FIGURE 4: *N*-Acetylgalactosamine inhibits the selective photosensitized labeling of a specific protein by FluNCS–lima bean lectin conjugate. Labeling of the 56-kDa target protein on S_{49} lymphoma cell membrane by FluNCS–lima bean lectin was carried out under incubation conditions in which different competing sugars were used to determine the specificity of the labeling process. Part I: FluNCS–lima bean lectin was bound to S_{49} lymphoma cell membranes as described under Methods and was subsequently treated with [^{125}I]INA and irradiated at 314 nm (direct photoactivation) as described (Figure 3, lane 1). Part II: As in part I except that membranes were irradiated at 480 nm. Part III: As in part II except that the FluNCS–lectin was preincubated for 2 h at room temperature with 0.25 M NAGA. Part IV: As for part III except that 0.25 M αMM substituted for NAGA. Following irradiation, membranes were washed and subjected to $NaDodSO_4$–PAGE on a 6% gel and autoradiographed as described in Figure 3. The autoradiograms were scanned by densitometry for quantitative evaluation of the labeling pattern. Peaks designated by small letters represent different membrane proteins labeled by [^{125}I]INA. Peak b is the 56-kDa protein which is selectively labeled by photosensitization from the FluNCS–lectin conjugate as is also seen in Figure 3, lane 4 (S_{49}). The numbers represent the relative area of each peak expressed as the percent of total optical density. Binding of FluNCS–lectin to membranes and all other details were as described under Methods. Densitometry was carried out by using a Bio-Rad 620 model video densitometer coupled to an IBM PC compatible microcomputer. The settings of the densitometer were as follows: filter frequency 2 lines/mm, enhancement frequency 0.05 lines/mm, and boost factor 10.

of [^{125}I]INA is given in Figure 4, part I, for comparison. Thus, the highly intensified labeling of protein band b seems to depend on its specific interaction with the chromophore-carrying ligand.

Discussion

We have recently reported that photosensitization of INA in biological membranes can be achieved by various sensitizers, including fluorescein derivatives, aminopyrene, trifluperazine, and rhodopsin. Since photosensitization is confined to the collisional range between INA and the sensitizer, it can be used to direct [^{125}I]INA labeling to chromophore-containing proteins in the membrane and in solution (Raviv et al., 1987). The present study was carried out to test the ability of this technique to elicit specific ligand-induced labeling of nearest-neighbor receptor molecules.

FluNCS–protein conjugates (FluNCS–BSA, FluNCS–STI), when mixed with membranes in suspension, induce radioactive labeling of membrane proteins by photosensitization of [^{125}I]INA. The interaction of the protein conjugates with the membrane seems to be random and nonspecific, since the labeling pattern elicited in S_{49} lymphoma cells is identical with the one obtained by direct irradiation of [^{125}I]INA at 314 nm (Figure 2), or by photosensitization of a randomly distributed chromophore sensitizer in the membrane (Raviv et al., 1987). This observation represents a general phenomenon, since the same results were also obtained with membranes from frog ROS, rat granulosa cells, mouse M2R melanoma, human erythrocytes, and bovine chromaffin cells (data not shown). Other FluNCS–protein conjugates used, like FluNCS–ovalbumin and FluNCS–avidin, produced similar results (data not shown). We believe that by adhering to the membrane in a nonspecific manner, these FluNCS–protein conjugates induce photosensitization of [^{125}I]INA in the lipid domain of the membrane by permitting the direct alignment of the donor–acceptor pair. Energy transfer takes place due to the intimate contact of the sensitizer with the membrane lipid. Furthermore, this interaction is probably enhanced by the lipophilic nature of the FluNCS moieties attached and also, to some extent, by the hydrophobicity of the proteins themselves. It is obvious that the use of such FluNCS–protein conjugates (i.e., FluNCS–BSA or FluNCS–STI) for sensitization also becomes a powerful method to selectively activate INA and exclusively label proteins in the plasma membrane of intact cells. This is in contrast to excitation at 314 nm, which activates INA directly and leads to nonselective labeling of all membranal proteins, including those of internal subcellular particles.

We wish to state that the methodologies for preparing membranes from both cell types used in this study do not take any special precautions to preserve their integrity or inside to outside orientation. In the case of ROS membranes, exposure of both the intradiscal face that contains the carbohydrate moiety of rhodopsin as well as the cytoplasmic face (Dratz & Hargrave, 1983) was desired. Thus, presentation of both sides of the membranes permitted random access of the reagents used.

FluNCS–lima bean lectin, which is known to interact with membranes at specific glucosyl binding sites (Galbraith & Goldstein, 1970), elicits random labeling only to a minor extent. Instead, it induces specific labeling of proteins in membranes from two different sources (Figures 3 and 4). The specific radioactivity of these proteins increased significantly, as can be seen in Figure 3 and also in Figure 4, by quantitative evaluation using densitometric analysis of the S_{49} lymphoma membrane labeling pattern.

The 88-kDa protein labeled in ROS is not detected at all by random labeling and is revealed only by photosensitized labeling with FluNCS–lima bean lectin. Thus, the predicted local amplification of [^{125}I]INA labeling, described schematically in Figure 1, part 3, can be demonstrated. The heterogeneous distribution of the FluNCS–protein conjugate is parallel to, and restricted by, the distribution of the specific binding sites in the membrane. The amplified radioactive labeling of these receptor proteins suggests that they are located in the vicinity of the FluNCS–lectin conjugate, close enough to permit their photosensitized tagging. This is further supported by the finding in S_{49} lymphoma membranes that *N*-acetylgalactosamine is able to block the labeling of the 56-kDa protein band (Figure 3, lane 4), also designated peak b (Figure 4, part II).

This result indicates that photosensitized [^{125}I]INA labeling of the target protein by the FluNCS–lectin conjugate is largely dependent on the specific interaction of the lectin with its respective binding sites on the membrane. However, the photosensitization process may induce labeling on unrelated proteins which may reside in the vicinity of the photosensitizer, since following energy transfer and activation of [^{125}I]INA the nitrene may diffuse for some distance before finally combining with a protein. Thus, the protein selectively labeled by the lectin may not necessarily represent the actual binding site.

Frog ROS proteins (Molday & Molday, 1979; Hamm & Bownds, 1986; Witt & Bownds, 1987) as well as mouse T-lymphocyte membrane proteins (Trowbridge et al., 1975; Gahmberg et al., 1976) have been extensively described in the literature. In neither case could we identify with certainty any major protein bands with the [^{125}I]INA-labeled proteins in the respective cell types. The identity, therefore, of these [^{125}I]INA-labeled proteins still remains to be elucidated.

Ligand-induced specific labeling of membrane proteins by this method is strongly affected by the nonspecific hydrophobic interaction between the ligand and the membrane and by the distance between the ligand–chromophore conjugate and the lipid phase of the membrane in which [^{125}I]INA distributed. Distances too large to permit collision between donor and acceptor, and subsequent exchange of energy, are likely to impair or even prevent labeling of membrane proteins by photosensitization. The lima bean lectin, being a large enough molecule (247 kDa), is most probably able to interact with its specific binding site, but at the same time with the membrane lipid (see also Figure 1, part 3). Thus, this lectin proved suitable for use in demonstrating of the principles of ligand-induced PSL of specific target proteins in membranes.

Photosensitized labeling was also applied to the study of interaction of proteins in solution using FluNCS–Con A as a ligand and ovalbumin as a target. [^{125}I]INA, which in aqueous solution attaches to the surface of FluNCS–Con A and ovalbumin, is photoactivated when the complex formed between these two proteins is irradiated at 480 nm. Consequently, both proteins became radioactively labeled. The labeling of ovalbumin could, however, be selectively eliminated by inclusion of αMM or glucose which binds to Con A, but not by galactose, which is not recognized by the lectin (data not shown).

The PSL technique thus introduces a novel way for nearest-neighbor labeling of proteins in biological systems that provides several advantages: (1) It can be carried out under extremely mild conditions without eliciting chemical cross-linking of components. (2) It can be done under conditions in which labeling will intensify progressively with time of irradiation. (3) It can be applied in the study of transient interactions between molecules. This approach offers additional possibilities and options in the study of molecular interactions in general, but also in examination of transient recognition processes involving cells, viruses, and liposomes, etc. Likewise, this technique can also be applied for general use in the targeting of photochemical processes in order to tag particular sites or cellular compartments (i.e., introduction of haptens, radioactive labels, etc.).

Acknowledgments

We gratefully acknowledge Rona Levin and Rachel Benjamin for their excellent secretarial assistance and Josepha Sole for her excellent technical assistance.

Registry No. [^{125}I]-INA, 66640-74-2; FluNCS-NHC$_{11}$, 117774-77-3.

References

Bayley, H. (1983) in *Laboratory Techniques in Biochemistry and Molecular Biology*. Vol. 12, Elsevier, Amsterdam, New York, and Oxford.

Bayley, H., & Knowles, J. R. (1977) *Methods Enzymol. 46*, 69–114.

Bayley, H., & Staros, V. J. (1984) in *Azides and Nitrenes, Reactivity and Utility* (Scriven, E. F. V., Ed.) pp 434–490, Academic, New York.

Bercovici, T., & Gitler, C. (1978) *Biochemistry 17*, 1484–1489.

Brunner, J. (1981) *Trends Biochem. Sci. (Pers. Ed.) 6*, 44–46.

Brunner, J., & Semenza, G. (1981) *Biochemistry 20*, 7174–7182.

Chowdry, R., & Westheimer, F. H. (1979) *Annu. Rev. Biochem. 48*, 293–325.

Czarnecki, J., Geaklen, R., & Haley, B. (1979) *Methods Enzymol. 56*, 642–653.

Davison, D. M., & Findlay, B. C. (1986) *Biochem. J. 234*, 413–420.

Dratz, E. A., & Hargrave, P. A. (1983) *Trends Biochem. Sci. (Pers. Ed.)*, 128–131.

Fedan, S. J., Hogaboom, G. K., & O'Donnel, J. P. (1984) *Biochem. Pharmacol. 33*, 1167–1180.

Gahmberg, C. G., Häyry, P., & Andersson, L. C. (1976) *J. Cell. Biol. 68*, 642–653.

Galbraith, W., & Goldstein, I. (1970) *FEBS Lett. 9*, 197–201.

Gitler, C., & Klip, A. (1974) in *Perspectives in Membrane Biology* (Estrada, O. S., & Gitler, C., Eds.) p 149. Academic, New York.

Guillory, R. J. (1979) *Curr. Top. Bioenerg. 9*, 268–414.

Guillory, R. J., & Jeng, S. J. (1977) *Methods Enzymol. 46*, 259–288.

Hamm, H. E., & Bownds, M. D. (1986) *Biochemistry 25*, 4512–4523.

Hazum, E. (1983) *Endocr. Rev. 4*, 352–362.

Ji, T. H. (1979) *Biochim. Biophys. Acta 559*, 39–69.

Ji, T. H., & Middaugh, C. R. (1980) *Biochim. Biophys. Acta 603*, 371–374.

Jørgensen, P. L., Karlish, S. J. D., & Gitler, C. (1982) *J. Biol. Chem. 257*, 7435–7442.

Klip, A., & Gitler, C. (1974) *Biochem. Biophys. Res. Commun. 60*, 1155–1162.

Klip, A., & Gitler, C. (1976) in *Mitochondria: Biogenesis, Structure and Function* (Packer, L., & Gomez-Dayou, E., Eds.) p. 315, Academic, New York.

Klip, A., Darszon, A., & Montal, M. (1976) *Biochem. Biophys. Res. Commun. 72*, 1350–1358.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

McDowell, H. J., & Kühn, H. (1977) *Biochemistry 16*, 4054–4060.

Middaugh, C. R., Vanin, E. F., & Ji, T. H. (1983) *Mol. Cell. Biochem. 50*, 115–141.

Molday, R. S., & Molday, L. L. (1979) *J. Biol. Chem. 254*, 4653–4660.

Peters, K., & Richards, F. M. (1977) *Annu. Rev. Biochem. 46*, 523–551.

Raviv, Y., Bercovici, T., Gitler, C., & Salomon, Y. (1984) *Biochemistry 23*, 503–508.

Raviv, Y., Salomon, Y., Gitler, C., & Bercovici, T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6103–6107.

Ross, A. H., Radhakrichman, R., Robson, R. J., & Khorana, H. G. (1982) *J. Biol. Chem. 257*, 4152–4161.

Ross, E. M., Maguire, M. E., Surgill, R. W., Biltonen, R. L., & Gilman, A. G. (1977) *J. Biol. Chem. 252*, 5761–5775.

Sidman, C. L., Bercovici, T., & Gitler, C. (1980) *Mol. Immunol. 17*, 1575–1583.
Tometsko, A. M., & Richards, F. M. (Eds.) (1980) *Ann. N.Y. Acad. Sci. 346*, 1–502.
Trowbridge, I. S., Ralph, P., & Bevan, M. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 157–161.
Witt, P. L., & Bownds, M. D. (1987) *Biochemistry 26*, 1769–1776.

Comparison of the Calcium Release Channel of Cardiac and Skeletal Muscle Sarcoplasmic Reticulum by Target Inactivation Analysis†

Susan G. McGrew,‡,§ Makoto Inui,‡,∥ Christopher C. Chadwick,‡ Robert J. Boucek, Jr.,⊥ Chan Y. Jung,# and Sidney Fleischer*,‡

Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235, Department of Pediatric Cardiology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232, and Biophysics Laboratory, Veterans Administration Medical Center, State University of New York at Buffalo, Buffalo, New York 14215

Received July 20, 1988; Revised Manuscript Received September 22, 1988

ABSTRACT: The calcium release channel of sarcoplasmic reticulum which triggers muscle contraction in excitation–contraction coupling has recently been isolated. The channel has been found to be morphologically identical with the feet structures of the junctional face membrane of terminal cisternae and consists of an oligomer of a unique high molecular weight polypeptide. In this study, we compare the target size of the calcium release channel from heart and skeletal muscle using target inactivation analysis. The target molecular weights of the calcium release channel estimated by measuring ryanodine binding after irradiation are similar for heart (139 000) and skeletal muscle (143 000) and are smaller than the monomeric unit (estimated to be about 360 000). The target size, estimated by measuring polypeptide remaining after irradiation, was essentially the same for heart and skeletal muscle, 1 061 000 and 1 070 000, respectively, indicating an oligomeric association of protomers. Thus, the calcium release channel of both cardiac and skeletal muscle reacts uniquely with regard to target inactivation analysis in that (1) the size by ryanodine binding is smaller than the monomeric unit and (2) a single hit leads to destruction of more than one polypeptide, by measuring polypeptide remaining. Our target inactivation analysis studies indicate that heart and skeletal muscle receptors are structurally very similar.

In excitation–contraction coupling, excitation at the sarcolemma leads to an elevated intrafiber Ca^{2+} concentration and thereby to muscle contraction. In vertebrate skeletal muscle, essentially all of the calcium for contraction is stored intracellularly within the sarcoplasmic reticulum (SR)[1] (Fleischer & Tonomura, 1985). Calcium is released from SR as a consequence of depolarization of the transverse tubule. This process is referred to as "depolarization induced calcium release". In heart, there are two pools of calcium: (1) extracellular calcium first enters the fiber through voltage-gated sarcolemmal calcium channels, and (2) the elevated Ca^{2+} then triggers calcium release from the SR compartment. This two-step process is referred to as "calcium induced calcium release" (Fabiato, 1983; Endo, 1977). A basic question to be resolved is whether the calcium release machinery in SR in heart is different from that in skeletal muscle.

The calcium release channels of SR from heart and skeletal muscle have recently been isolated and identified in molecular terms (Inui et al., 1987a,b; Lai et al., 1988a,b; Hymel et al., 1988a,b; Fleischer & Inui, 1988; Imagawa et al., 1987). The breakthrough was based on two key advances: (a) the isolation of a terminal cisternae fraction of SR containing well-defined feet structures which served as a test system (Saito et al., 1984) and (b) the finding that ryanodine is a specific ligand for the calcium release channel of SR (Fleischer et al., 1985). The isolated ryanodine receptor from heart and skeletal muscle consists of an oligomer of a single high molecular weight polypeptide (Inui et al., 1987b; Lai et al., 1988a,b; Imagawa et al., 1987). The receptor has been identified morphologically as the foot structure (Inui et al., 1987a,b) which spans the triad or dyad junction (Franzini-Armstrong & Nunci, 1983) between terminal cisternae and transverse tubules or plasmalemma. The identity of the ryanodine receptor as the channel was achieved by reconstituting it into bilayers. Channel gating behavior was obtained, and the response to specific ligands reflected the permeability changes characteristic of terminal cisternae of SR (Hymel et al., 1988a,b; Lai et al., 1988a,b).

In this study, we compare structural characteristics of the calcium release channel from heart and skeletal muscle by target inactivation analysis.

Materials and Methods

All chemicals were reagent grade or the best available and were prepared in deionized water. Protein was measured according to the method of Lowry et al. (1951) using bovine plasma albumin as the standard. Cardiac microsomes were

† This work was supported in part by grants from the National Institutes of Health (DK 14632 and HL 32711), by the Muscular Dystrophy Association of America, and by a Biomedical Research Support grant from the National Institutes of Health administered by Vanderbilt University.

‡ Department of Molecular Biology, Vanderbilt University.
§ Recipient of a fellowship from the American Heart Association, Middle Tennessee Chapter.
∥ Investigator of the American Heart Association, Tennessee Affiliate.
⊥ Department of Pediatric Cardiology, Vanderbilt University School of Medicine.
State University of New York at Buffalo.

[1] Abbreviations: SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; SR, sarcoplasmic reticulum; HMW polypeptide, high molecular weight polypeptide.

0006-2960/89/0428-1319$01.50/0