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Covalent Attachment of Sulfhydryl-Specific, Electron Spin Resonance Spin-Labels to Fab′ Fragments of Murine Monoclonal Antibodies That Recognize Human Platelet Membrane Glycoproteins. Development of Membrane Protein Specific Spin Probes†

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ABSTRACT: A general method for the production of high-affinity, nitroxide-labeled, protein-specific spin probes is described in this paper. Fab′ fragments are generated from protein-specific, murine monoclonal antibodies by pepsin digestion and mild reduction with cysteine. The free sulfhydryl group located in the carboxy-terminal region of these molecules and produced de novo by this manipulation is then alkylated by reaction with 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO-maleimide), thereby generating spin-labeled Fab′ fragments of these monoclonal antibodies. Two prototypic monoclonal antibodies were tested, each specific for a different integral membrane glycoprotein of human blood platelets. The results indicate that Fab′ spin probes generated by this method retain the ability to bind to these glycoproteins within the membrane of intact platelets. These reagents thus represent probes that can be generally used to monitor integral membrane protein mobility on the surface of the intact cell.

The investigation of dynamic interactions between integral membrane proteins in situ is limited by an inability to selectively label a given protein without its prior removal from the membrane and subsequent reinsertion into native or synthetic membranes. Protein labeling methods that do not require cell membrane disruption, on the other hand, are largely nonspecific. Indirect methods such as chemical cross-linking or immunoelectron microscopy provide only an approximation of protein–protein interactions over a fairly large time span, at best, no less than a few minutes, and cannot distinguish functionally relevant interactions from random collisions. Millisecond cross-linking of membrane proteins in situ using photoactivatable reagents has met with limited success due to the inefficiency of photoactivation in cell-rich media and, once again, a general lack of specificity (Ji, 1977; Keihm & Ji, 1977; Bayley & Knowles, 1980; Rotman & Pribluda, 1982). Thus, the ability to draw meaningful conclusions concerning the mobility and/or associations of membrane proteins in situ in real time awaits the development of noninvasive protein-specific reagents using detection systems by which events occurring within the millisecond to nanosecond time range can be monitored.

In this paper, we introduce a general method for the production of high-affinity, nitroxide-labeled, protein-specific spin probes, namely, Fab′ fragments of glycoprotein-specific, murine monoclonal antibodies that have been alkylated in their carboxy-terminal region with 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO-maleimide). We show that such reagents can be easily produced in high yield, are stable indefinitely during storage at −80 °C, and retain the ability to bind to their glycoprotein antigens with little or no change in original affinities.

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Two prototypic monoclonal antibodies are described: C7E10, specific for human platelet membrane glycoprotein (GP) Ib, the receptor for plasma von Willebrand factor that mediates platelet adhesion to the vessel wall (Nugent et al., 1984), and AP3, specific for GPIIIa, one component of the receptor for plasma fibrinogen that mediates platelet cohesion (Newman et al., 1985).

Materials and Methods

Purification of Monoclonal IgG,[1] *Production of Fab' Fragments, and Spin-Labeling of Fab'.* Each murine IgG was purified from ascites fluids by a method determined to provide optimum yields for that particular antibody. C7E10 IgG was purified by adsorption onto staphylococcal protein A–Sepharose and elution with 0.1 M citrate, pH 4.4, as described (Nugent et al., 1984), while AP3 IgG was purified by caprylic acid precipitation and DEAE-Affi-Gel Blue chromatography (Newman et al., 1985). Murine IgG subclass analyses indicated that C7E10 is an IgG2a, while AP3 is an IgG1. The production of Fab' fragments by pepsin digestion and limited reduction is based upon the procedure of Lombardo et al. (1985). Purified IgG (1.0 mg) was dialyzed into 0.12 M acetate and 0.15 M NaCl, pH 3.5; then $F(ab')_2$ fragments were generated by addition of 0.01 mg of pepsin (Worthington Biochemical Corp., Freehold, NJ) in the same buffer and incubation at 37 °C for 90 min. The mixture was dialyzed against 0.5 M Tris-HCl, pH 8.0, at ambient temperature with two buffer changes (each 0.5 L). Fab' fragments were produced by limited reduction, specifically, incubation in the presence of 0.01 M L-cysteine (added as free base, final concentration) in 0.5 M Tris-HCl, pH 8.0 at 37 °C, for 1 h. All subsequent operations were performed with buffers that had been evacuated by aspiration and bubbled with nitrogen gas. Fab' fragments were dialyzed against three changes (each 0.5 L) of 0.03 M Tris-HCl and 0.15 M NaCl, pH 7.4. To 1.0 mL of this preparation was added 0.1 mL of 0.02 M TEMPO-maleimide in 50% acetonitrile, and the mixture was incubated at 37 °C for 3 h, dialyzed against three or more changes (1.0 L each) of the above Tris buffer, pH 7.4, and finally concentrated to a volume of 0.1 mL with a ProDiCon apparatus (Bio-Molecular Dynamics, Beaverton, OR). Spin-labeled Fab' fragments were stored in 0.025–0.5-mL aliquots at −80 °C.

ESR Measurements. All ESR spectra were recorded with a Varian Century line 9-GHz spectrometer equipped with a Varian temperature controller and a digital thermometer (Fluke Model 2100A) as previously described (Lai et al., 1984). The incident microwave power was 10 mW. The field sweep was 100 G, and the modulation amplitude was 2.0 G. The amount of label bound to antibody molecules was determined by double integration of ESR spectra with a digital signal analyzer (Tracor Northern NS-570A). During these quantitative measurements, the incident microwave power and modulation amplitude were 1 mW and 1 G, respectively. Effective rotational correlation times were determined by using the equation derived by Stone et al. (1965)

$$1/\tau = [(3.6 \times 10^9)/\Delta H_0(H_0/H_{-1})^{1/2} - 1]\ s^{-1}$$

where ΔH_0 is the peak-to-peak width of the central field line (in gauss) and H_0 and H_{-1} are peak-to-peak heights of the central field and high field lines, respectively.

Platelet Isolation. Platelets were isolated from whole blood anticoagulated with ACD and separated from plasma proteins by differential centrifugation as previously described (Kunicki et al., 1985). When prepared by this method and stored at ambient temperature for less than 5 h, platelets retained the ability to bind fibrinogen and undergo aggregation in response to physiologic agonists, e.g., thrombin, ADP, or epinephrine.

Ristocetin-Induced Platelet Agglutination. A microtiter tray assay for the inhibition of ristocetin-induced agglutination of formalin-fixed platelets was performed essentially as described by Montgomery et al. (1983). Each well contained 100 μL of formalin-fixed platelets (2×10^8/mL) incubated for 30 min in the presence of 10 μL of buffer (0.03 M Tris, 0.15 M NaCl, pH 7.4) containing monoclonal IgG or spin-labeled Fab'. Normal plasma (15 μL) and 5 μL of ristocetin (final concentration 1.2 mg/mL) were added, and the microtiter tray was agitated gently for 30–60 min at ambient temperature. The presence or absence of agglutination was then recorded photographically.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). One-dimensional SDS–PAGE using the discontinuous buffer system of Laemmli (1970) was performed as described (Kunicki et al., 1985) with a 7–12% exponential gradient polyacrylamide resolving slab gel (for nonreduced samples) or a 10% polyacrylamide resolving slab gel (for reduced samples). Molecular weights were determined by comparison to the mobilities of molecular weight standards (Bio-Rad Laboratories, Richmond, CA), including myosin (mol wt 200 000), β-galactosidase (mol wt 117 000), phosphorylase *b* (mol wt 94 000), bovine serum albumin (mol wt 69 000), ovalbumin (mol wt 43 000), and carbonic anhydrase (mol wt 31 000). Complete reduction of disulfide bonds for SDS–PAGE was affected by prior incubation of the protein suspension in the presence of 5% (v/v) 2-mercaptoethanol and 1% (w/v) SDS for 1 h at 37 °C.

Results

Since previous experience had shown that the optimum pH for maximum digestion of monoclonal antibodies by pepsin can vary significantly, within a range from 3.0 to 4.5 (Lombardo et al., 1985), preliminary studies were performed to determine the optimum pH at which the monoclonal antibodies C7E10 and AP3 were maximally digested. pH 3.5 was found to be optimum for both antibodies. Additional preliminary experiments were conducted to determine the optimum time of digestion as well as the optimum enzyme-to-substrate ratio for each monoclonal antibody. Under the conditions employed in this study, cleavage by pepsin was complete within 90 min at 37 °C when the enzyme-to-substrate ratio was greater than or equal to 1% (weight of pepsin/weight of protein substrate).

By SDS–PAGE (Figure 1), purified C7E10 IgG gave a single major protein band under nonreduced conditions (lane 1). Upon reduction, two heavy chains and two light chains were detected (Figure 1, lane 4). After digestion with pepsin, no intact IgG remained, and two protein bands in the molecular weight range of 95 000–105 000, a size consistent with that of $F(ab')_2$ fragments, were essentially the sole components detected under nonreduced conditions (Figure 1, lane 2). Similar results were obtained with AP3 as substrate (data not shown). Upon reduction of $F(ab')_2$ in SDS–PAGE, protein bands corresponding to intact light chains and the amino-terminal segments of digested heavy chains were detected (Figure 1, lane 5). Following carefully controlled reduction of these $F(ab')_2$ fragments with 10 mM cysteine, three protein bands with molecular weights of Fab' fragments of 45 000–

[1] *Abbreviations:* ACD, acid–citrate–dextrose anticoagulant; ADP, adenosine 5'-diphosphate; ESR, electron spin resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide; DEAE, diethylaminoethyl; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane.

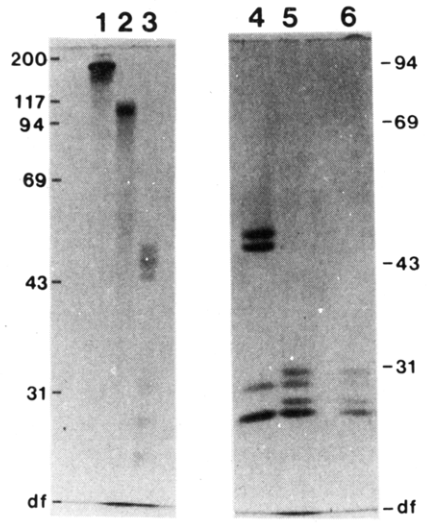

FIGURE 1: SDS–PAGE of purified C7E10 and fragments produced by pepsin digestion, reduction, and alkylation. Six micrograms of sample protein was separated in a 7–12% exponential gradient polyacrylamide resolving gel under nonreduced conditions (lanes 1–3) or in a 10% polyacrylamide resolving gel under reduced conditions (lanes 4–6) as described under Materials and Methods. The gels depicted here were stained with Coomassie blue R to visualize protein bands. Protein samples analyzed were (lanes 1 and 4) intact, purified IgG, (lanes 2 and 5) $F(ab')_2$ fragments derived from intact IgG by digestion with pepsin, and (lanes 3 and 6) Fab′ fragments produced by controlled reduction and alkylation in the presence of a 100-fold molar excess of TEMPO-maleimide (in this case, the [^{14}N]nitroxide label). The mobility of molecular weight standards is indicated next to each gel together with their apparent molecular weights ($\times 10^{-3}$). df is the dye front.

55 000 were detected by SDS–PAGE (Figure 1, lane 3), composed of intact light chains and digested heavy chain remnants (Figure 1, lane 6). The mobilites of Fab′ fragments subsequently alkylated either by addition of a greater than 1000-fold molar excess of NEM or by addition of a 100-fold molar excess of TEMPO-maleimide were identical.

The ESR spectrum of the [^{14}N]nitroxide maleimide spin-label alone in buffer (Figure 2A) exhibits three sharp lines of about equal intensity with an isotropic hyperfine constant of 16.0 G and an effective rotational correlation time of 2.1×10^{-11} s, typical of small nitroxides undergoing a fast isotropic tumbling motion in solution. The monoclonal Fab′ fragments chemically modified with this maleimide spin-label generate ESR spectra such as those depicted in Figure 2B (for C7E10) and in Figure 2D (for AP3). A significant reduction in probe motion is evident upon covalent attachment to the monoclonal Fab′ fragments, and the effective rotational correlation times for spin-labeled C7E10 and spin-labeled AP3 were 3.0×10^{-10} and 2.0×10^{-10} s, respectively. The molar ratio of the bound nitroxide group to the Fab′ molecule was 1.14 for C7E10 and 1.16 for AP3, indicating that, on the average, one free sulfhydryl group per Fab′ fragment had been produced by reduction and then alkylated with the maleimide spin probe by using the procedure outlined in this paper. Equivalent results were obtained with [^{15}N]nitroxide maleimide spin-label using either monoclonal antibody (data not shown).

To determine whether spin-labeled Fab′ fragments retain the ability to bind to platelets, we looked at the effect of platelet binding upon the ESR spectra. As shown in Figure 2C (for C7E10) and Figure 2E (for AP3), a further immobilization of the spin probe was observed when it became platelet bound, reflected in a further increase in the effective rotational correlation times to 4.2×10^{-10} (for C7E10) and 3.9×10^{-10} s (for AP3).

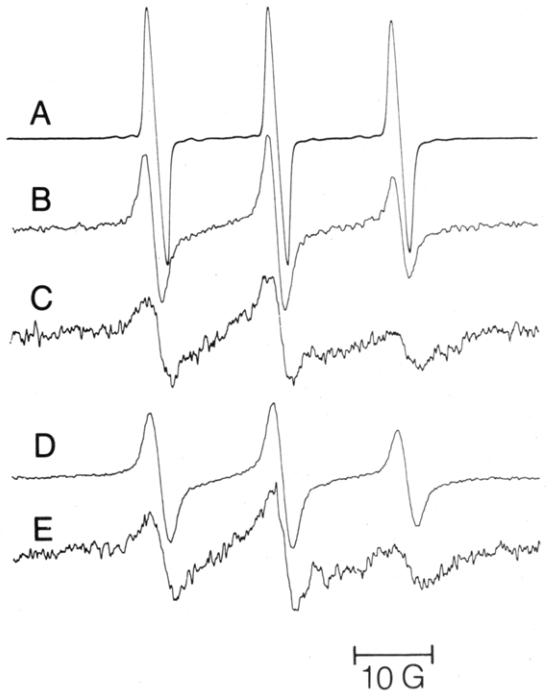

FIGURE 2: ESR spectra. The spectrum derived from the free [^{14}N]nitroxide maleimide spin probe in buffer (A) is compared to those obtained with spin-labeled C7E10 Fab′ (B) and spin-labeled AP3 Fab′ (D). Washed platelets were incubated with saturating levels of these spin-labeled Fab′ fragments, washed exhaustively, and then analyzed. The spectra of the platelet-bound, spin-labeled Fab′ fragments are shown: (C) C7E10; (E) AP3. Instrument settings: in (A) the modulation amplitude is 1 G, and the microwave power is 10 mW; in (B–E) these values are 2 G and 10 mW, respectively. Other experimental conditions and scan parameters are described under Materials and Methods.

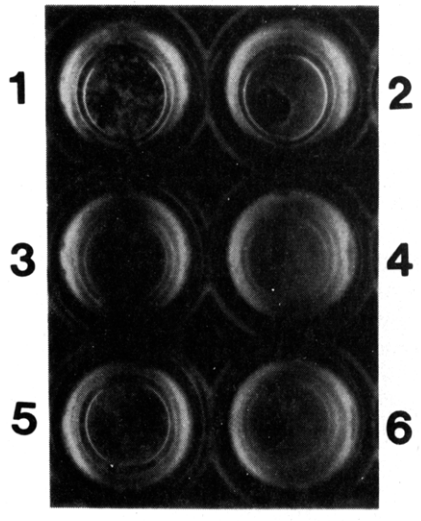

FIGURE 3: Inhibition of ristocetin-induced agglutination of formalin-fixed platelets by C7E10 and AP3 IgG and spin-labeled Fab′ fragments derived therefrom. In the absence of monoclonal IgG or spin-labeled Fab′ fragments (well 1), normal agglutination of platelets was observed. Prior incubation in the presence of 5 μg of spin-labeled AP3 Fab′ (well 2) had no inhibitory effect. On the other hand, prior incubation in the presence of 1 or 2 μg of spin-labeled C7E10 Fab′ (wells 3 and 4, respectively) completely inhibited agglutination. Intact C7E10 IgG at 0.5 μg (well 5) did not inhibit, while complete inhibition was again observed with 1 μg of intact C7E10 IgG (well 6). The Fab′ fragments used in the experiment depicted here were labeled with the [^{14}N]nitroxide maleimide probe.

The affinity of the monoclonal antibodies modified in this manner was essentially unperturbed. For example, as shown in Figure 3, spin-labeled Fab′ fragments of C7E10 retained the ability to inhibit the ristocetin-induced agglutination of

formalin-fixed platelets, and the minimum concentration of spin-labeled Fab′ of C7E10 required to detect inhibition of agglutination was essentially identical with that concentration required of intact C7E10 IgG (roughly 1.0 μg per 10^8 platelets). Thus, no significant loss of C7E10 affinity resulted from this modification.

Discussion

In this study, we demonstrate a simple, straightforward method for the covalent attachment of spin-labels onto the primary structure of an antibody molecule. Previous studies of spin-labeled antibody molecules used sialic acid specific reagents to attach the probe to the terminal carbohydrate of oligosaccharide chains located predominantly in the hinge region of the IgG molecule (Nezlin et al., 1978; Willan et al., 1977; Winkelhake et al., 1984). Several studies have shown that IgG carbohydrate moieties are not directly involved in the antigen binding function of the antibody molecule (Koide et al., 1977; Winkelhake et al., 1980; Van Schravendijk & Dwek, 1981). Thus, while such probes provide valid information about the immediate environment of the carbohydrate-containing region of the molecule and report conformational change of the oligosaccharide chains, they are less than ideal for monitoring the interactions of an antibody molecule with its antigen or the subsequent motion of the antigen–antibody complex. Our approach, outlined in this paper, puts the spin-label onto the side chain of a cysteine residue that is first generated by the controlled reduction of the disulfide normally joining the IgG heavy chain subunits.

Chemical modification of protein sulfhydryl groups with TEMPO-maleimide has been shown to provide a very sensitive means to monitor protein motion, for example, in the case of fibronectin (Lai et al., 1984). Since the maleimide spin-label is covalently attached to the primary structure of the molecule, its mobility is significantly restricted, as demonstrated by Lai et al. (1984) for fibronectin and as described for Fab′ fragments of IgG in this paper. In the case of IgG Fab′, the label is attached at a position on the molecule diametrically opposite to the region involved in antigen binding. Thus, it is also unlikely to interfere with antigen-specific antibody binding.

The procedure outlined in this paper can be used to generate protein-specific spin probes that selectively bind to integral membrane proteins in situ. This represents the first instance that spin-label methods are used to study the motion of a single protein component on the surface of intact cells. ESR studies of membrane proteins to date have been restricted to the use of probes that nonselectively label any reactive membrane protein, for example, labeling of erythrocyte membrane (glyco)proteins in situ with TEMPO-maleimide and Tempamine [see Butterfield (1982) for a review] or labeling of purified membrane proteins ex situ and subsequent insertion of the labeled proteins into synthetic membrane vesicles (Davoust et al., 1983). In the former case, the lack of specificity for a single membrane protein obviously limits the interpretation of results, while in the latter case, the requirement for isolation, purification, derivatization, and then reinsertion of the specifically labeled protein must ultimately have a significant effect upon its conformation and molecular motion.

Several biophysical methods [reviewed by Vaz et al. (1984)] have been applied to the measurement of lipid mobility on the surface of the intact cell including fluorescence recovery following photobleaching, eximer formation, pulsed gradient nuclear magnetic resonance, electron spin resonance techniques that employ line-width analysis, and, very recently, electron–electron double resonance (ELDOR) methods. The use of ^{14}N, ^{15}N spin-label pairs in the ELDOR method represents a marked improvement since the problem of intramolecular contributions to the ELDOR effect caused by nitrogen nuclear relaxation is essentially eliminated (Feix et al., 1984). This improved ELDOR method, using 16-doxylstearate spin-label pairs, was employed to measure the lateral diffusion constant D of lipids in the plasma membrane of intact human platelets, thereby demonstrating a senescence-dependent increase in D (from 1.0×10^{-8} to 2.6×10^{-8} cm^2/s at 37 °C) during platelet storage in vitro (Lai et al., 1986). This paper represented the first measurement of D in blood platelets and the first demonstration of an effect of aging in vitro upon platelet membrane lipid mobility. The development of protein-specific spin probes will enable one to use the spin-label pair ELDOR method to directly measure the mobility of proteins on the surface membrane of intact cells. With a judicious choice of spin-probe combinations, one can also directly measure protein–protein collision frequencies within the cell membrane.

Protein-specific antibody spin probes may also prove to be valuable nitroxide paramagnetic contrast agents for in vivo magnetic resonance (MR) imaging. Because of their paramagnetic properties, nitroxide spin-labels enhance the relaxation rates of water protons and are potential contrast agents for MR imaging (Brasch, 1983; Slane et al., 1986). For example, maleimide spin-probe antibodies that recognize tumor-specific antigens could be used to localize such tumors, or focal areas of thrombosis could be visualized by using spin-probe antibodies that react with antigens only expressed on activated platelets. These potential applications for this new technology warrant further investigation.

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Amplification of Phosphodiesterase Activation Is Greatly Reduced by Rhodopsin Phosphorylation†

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ABSTRACT: In the vertebrate rod outer segment (ROS), the light-dependent activation of a GTP-binding protein (G-protein) and phosphodiesterase (PDE) is quenched by a process that requires ATP [Liebman, P. A., & Pugh, E. N. (1979) *Vision Res. 19*, 375–380]. The ATP-dependent quenching mechanism apparently requires the phosphorylation of photoactivated rhodopsin (Rho*); however, a 48-kilodalton protein (48K protein) has also been proposed to participate in the inactivation process. Purified species of phosphorylated rhodopsin containing 0, 2, or ≥4 (high) phosphates per rhodopsin (PO_4/Rho) were reconstituted into phosphatidylcholine (PC) vesicles and reassociated with a hypotonic extract from isotonically washed disk membranes that were depleted of 48K protein; PDE activation, in response to bleaching from 0.01% to 15% of the rhodopsin present, was measured. PDE activity was reduced by at least 30% at high fractional rhodopsin bleaches and by greater than 80% at low fractional rhodopsin bleaches in high PO_4/Rho samples when compared to the activity measured in 0 PO_4/Rho controls. A phosphorylation level of 2 PO_4/Rho produced PDE activities that were intermediate between 0 PO_4/Rho and high PO_4/Rho samples at low bleaches, but were identical with the 0 PO_4/Rho samples at high rhodopsin bleaches. Rhodopsin phosphorylation is thus capable of producing a graded inhibition of light-stimulated PDE activation over a limited range of (near physiological) bleach levels. This effect becomes less pronounced as the bleach levels approach those that saturate PDE activation. These results are consistent with increasing levels of phosphorylation, producing a reduction of the binding affinity of G-protein for Rho*.

The outer segment of the vertebrate rod cell contains a number of enzymes that are activated in a light-dependent manner. Each of these enzymatic activities are temporally linked to the photobleaching of the visual pigment rhodopsin. An active form of bleached rhodopsin, Rho*, catalyzes the exchange of GTP for previously bound GDP on a GTP-binding protein (G-protein)[1] (Fung & Stryer, 1980). The G-protein, which posesses a slow intrinsic, GTPase activity (Godchaux & Zimmerman, 1979; Kuhn, 1980; Liebman & Pugh, 1981), is then capable of activating a cGMP-specific phosphodiesterase (PDE); this latter function is lost when the bound GTP is hydrolyzed to GDP.

Bleached rhodopsin is also a substrate for phosphorylation by rhodopsin kinase (Kuhn, 1978; Shichi & Somers, 1978; Liebman & Pugh, 1979; Wilden & Kuhn, 1982; Kuhn & Wilden, 1982; Aton et al., 1984; Sitaramayya, 1986). A considerable body of evidence supports Liebman's hypothesis that the phosphorylation of rhodopsin is the key regulatory step in a rapid, ATP-dependent inactivation of Rho* (Liebman & Pugh, 1979, 1981; Sitaramayya & Liebman, 1983a,b; Aton & Litman, 1984; Miller & Dratz, 1984). A recent report by Sitaramayya (1986) suggests that only 1–2 PO_4/Rho are required for the rapid, ATP-dependent inactivation of Rho*. Other reports, however, suggest that rhodopsin phosphorylation alone is not sufficient for the rapid inactivation of Rho*. Kuhn and co-workers (Kuhn et al., 1984; Pfister et al., 1985) have reported that the G-protein binds to both "phosphorylated Rho*" and unphosphorylated Rho*. In addition, they report that an endogenous 48-kilodalton protein (48K protein) binds

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; cGMP, guanosine cyclic 3′,5′-phosphate; ROS, rod outer segment(s); Rho, rhodopsin; PDE, phosphodiesterase; G-protein, also called GTP-binding protein, GTPase, and transducin; 48K protein, 48-kilodalton protein; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; OG, octyl β-D-glucoside; con A, concanavalin A–Sepharose; KIU, Kallikrein inhibitor unit; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PO_4/Rho, phosphates per rhodopsin; PC, phosphatidylcholine.

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