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Papain Fragmentation of the (Na^+,K^+)-ATPase β Subunit Reveals Multiple Membrane-Bound Domains†

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ABSTRACT: Purified dog kidney (Na^+,K^+)-ATPase was reacted with tritiated sodium borohydride after treatment with neuraminidase and galactose oxidase. This procedure did not affect the ATPase activity of the enzyme, and all of the covalently bound radioactivity was found in the β subunit (M_r 54 000). Papain digestion of the tritiated enzyme produced two labeled fragments of M_r 40 000 and 16 000. Further proteolysis generated an M_r 31 000 peptide from the larger fragment. Unlike the tryptic and chymotryptic sites of the α subunit, the sites of papain hydrolysis were insensitive to conformations of the (Na^+,K^+)-ATPase. Determination of the NH_2-terminal sequences was used to arrange the fragments within the linear map of the β chain. Finally, none of the labeled peptides was released from the membrane under nondenaturing conditions. These results are consistent with a model of the β subunit containing a 40 000-dalton NH_2-terminal piece and a 16 000-dalton COOH-terminal piece. Both fragments have extracellularly exposed carbohydrate and at least one membrane-bound domain.

The (Na^+,K^+)-ATPase couples the hydrolysis of ATP to the active transport of Na^+ and K^+ across the plasma membrane [for reviews, see Forgac & Chin (1984), Jorgensen (1982), and Cantley (1981)]. It consists of two polypeptide chains, α of M_r 100 000 and β of M_r 60 000.[1] Proteolytic enzymes and chemical labeling reagents have been used to identify cytoplasmic, extracellular, and membrane-embedded regions of the protein. The α chain traverses the lipid bilayer many times and extends into both aqueous compartments (Chin & Forgac, 1983; Jorgensen et al., 1982; Farley et al., 1980).

Less is known about the disposition of the β chain with respect to the lipid bilayer. The small subunit contains covalently bound carbohydrate, and the mass of the protein portion has been estimated at 30 000–50 000 daltons (Fambrough & Bayne, 1983; Craig & Kyte, 1980). Lipid-soluble reagents and a digitoxin derivative have been shown to react with the β subunit (Jorgensen & Brunner, 1983; Jorgensen et al., 1982; Montecucco et al., 1981; Farley et al., 1980; Hall & Ruoho, 1980). Giradet et al. (1981) have described an antiserum apparently directed toward cytoplasmic determinants of the β subunit. All of these studies have dealt only with the intact β polypeptide. To obtain greater resolution of the protein structure, as has been achieved with the α chain, it would be advantageous to be able to examine smaller regions whose locations within the native molecule are known.

In this work, papain is used to fragment the membrane-bound dog kidney (Na^+,K^+)-ATPase. The sites of proteolysis and the papain fragments are characterized. These results and the data presented by other workers are incorporated into a model of the structure of the β subunit with respect to the membrane.

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[1] Apparent molecular weights are based on mobilities in SDS–polyacrylamide gel electrophoresis.

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Materials and Methods

(Na^+,K^+)-ATPase was prepared as purified membranes from dog kidneys (Jorgensen, 1974). The final specific activity was 20–30 μmol of ATP min^{-1} (mg of protein)$^{-1}$ at 37 °C and was >95% ouabain inhibitable. Papain (type III), neuraminidase (type X), and galactose oxidase (type V) were from Sigma. Sodium dodecyl sulfate (SDS[2])–polyacrylamide gel electrophoresis reagents were from Bio-Rad. NaB^3H_4 (5.6 Ci/mmol), Aquasol, and EN^{3}HANCE were from New England Nuclear.

Carbohydrate labeling was performed following Steck & Dawson (1974). (Na^+,K^+)-ATPase membranes were diluted to 1 mg/mL in 25 mM imidazole (pH 6.8) and incubated with neuraminidase (0.16 unit/mg of ATPase) and galactose oxidase (0.92 unit/mg of ATPase) for 45 min at 23 °C. Then NaB^3H_4 (2.5 mCi/mg of ATPase) was added and allowed to react for 45 min. The reaction was stopped by the addition of 5 volumes of ice-cold buffer, and the mixture was centrifuged for 45 min at 200000*g*. The membrane pellet was washed once with buffer and resuspended to 1 mg/mL.

Papain digestions were carried out for 30 min at 37 °C in a buffer of 25 mM imidazole (pH 6.8) containing 5 mM EDTA, 20 mM 2-mercaptoethanol, and 0.1 M NaCl except as noted. The reactions were quenched either by boiling in SDS gel sample buffer or by addition of *N*-ethylmaleimide (in excess over 2-mercaptoethanol) followed by sample buffer.

SDS–polyacrylamide gel electrophoresis was by the method of Laemmli (1970). Analytical gels were stained with Coomassie blue or fixed and prepared for fluorography using EN^{3}HANCE. For scintillation counting of radioactive bands, dried gel slices were digested overnight at 55 °C in 0.4 mL of 30% H_2O_2 and cooled prior to addition of Aquasol. In preparative gels, protein bands were located by staining guide strips. The appropriate gel slices were excised and soaked in 5 volumes of 50 mM NH_4HCO_3 and 0.1% SDS for 24 h at 37 °C with one change. The combined eluates were filtered through glass wool, lyophilized, and dialyzed against 2 L of 50 mM NH_4HCO_3 and 0.025% SDS for 24 h at 23 °C with one change. The purified peptides (0.6–1.6 nmol by amino acid analysis) were sequenced by Dr. David Andrews and Willian Lane of the Protein Chemistry Facility at Harvard University using an Applied Biosystems 470A microsequencer (Hunkapiller et al., 1983) with resolution of the PTH-amino acids by HPLC (Hawke et al., 1982).

Results and Discussion

The straightforward approach to proteolysis of the (Na^+,K^+)-ATPase β subunit would use natural membranes containing the native, purified protein. Peptide fragments would be resolved by electrophoresis on SDS–polyacrylamide gels and located by staining with Coomassie blue. There are three reasons why this approach is not feasible. First, the α and β subunits have not yet been separated under nondenaturing conditions. Second, the α subunit is digested more rapidly than the β subunit. This means that peptide fragments derived from the β chain will be mixed in with those from the α chain. Third, in SDS gel electrophoresis, the β chain migrates as a diffuse band that is poorly stained by Coomassie blue. In order to investigate the proteolytic structure of the native β subunit, it is necessary to have a sensitive method for selectively identifying peptides derived from the β chain.

[2] Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PTH, phenylthiohydantoin; HPLC, high-pressure liquid chromatography.

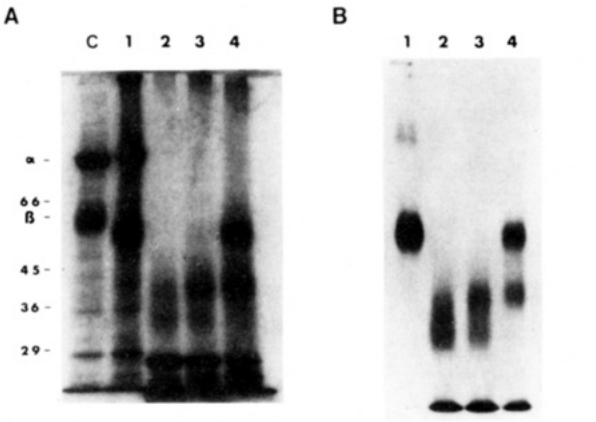

FIGURE 1: Papain digestion of tritium-labeled (Na^+,K^+)-ATPase. Purified (Na^+,K^+)-ATPase was reacted with NaB^3H_4, incubated with papain, and electrophoresed on a 10% SDS gel as described under Materials and Methods. (A) Coomassie blue stain: lane C, 21 μg of purified ATPase; lanes 1–4, 42 μg of purified ATPase reacted with 0, 14, 5.6, and 1.4 μg of papain. (B) Fluorography: lanes 1–4, one-tenth of the samples loaded in lanes 1–4 of (A). The film was exposed for 2 days at −70 °C.

Enzymatic labeling of galactose residues was studied because the dog kidney β subunit is a sialoglycoprotein and the α subunit is devoid of carbohydrate (Omori et al., 1983). Terminal sialic acid residues are often linked to a penultimate galactose (Hubbard & Ivatt, 1981).

The membrane-bound ATPase was treated with neuraminidase and galactose oxidase prior to reduction of the C-6 aldehyde with tritiated sodium borohydride. In Figure 1A (lanes C and 1), it can be seen that this protocol produces a β chain of increased mobility with no change in the apparent molecular weight of the α chain. The ATPase activity is not affected; this labeled preparation had a specific activity of 22 μmol of ATP min^{-1} (mg of protein)$^{-1}$ at 37 °C. The shift in the apparent molecular weight of β from 60000 to 54000 occurred on incubation with neuraminidase alone, in agreement with Omori et al. (1983). These data indicate that the movement of the β chain is due to removal of sialic acid and not to contaminating proteases. Furthermore, the concentration of sodium borohydride was kept relatively low, 0.45 mM, to minimize chemical cleavage of peptide bonds.

The covalently incorporated radioactivity is confined to the glycoprotein and to a faint band that does not comigrate with α but does migrate in the position expected for a β dimer (Figure 1B, lane 1). This dimer might be a noncovalent association of β chains that is detected only because of the sensitivity of fluorography and because the samples were solubilized at 23 °C. The dimer might also be a covalent adduct between the aldehyde of an oxidized molecule and a reactive amino group of another molecule. This reaction would lead to incorporation of radioactivity on reduction of the Schiff's base. In control experiments, very little radioactivity was incorporated in the absence of enzymes, galactose oxidase increased this level 4-fold, and the combination of neuraminidase and galactose oxidase further increased it by a factor of 3. All of these results are consistent with specific labeling of galactose residues and with previous studies of this method (Gahmberg, 1976; Steck & Dawson, 1974).

Hydrolysis of the α and β subunits by decreasing concentrations of papain is shown in lanes 2–4 of Figure 1A (Coomassie blue) and Figure 1B (fluorography). The large subunit is rapidly degraded to peptides of M_r ≤20000. The small subunit generates two fragments of M_r 40000 and 31000, along with labeled material that runs at the dye front on a 10%

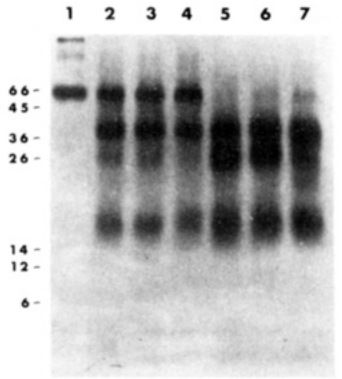

FIGURE 2: Papain digestion in the presence of ligands of the (Na^+,K^+)-ATPase. Purified, labeled (Na^+,K^+)-ATPase was incubated with papain in the following ligand environments and then electrophoresed on a 15% SDS gel as described under Materials and Methods. Lane 1, 4 μg of ATPase, no ligands; lanes 2–4, 5 μg of ATPase reacted with 0.5 μg of papain; lanes 5–7, 5 μg of ATPase reacted with 2.5 μg of papain; lanes 2 and 5, 80 mM KCl; lanes 3 and 6, 80 mM NaCl; lanes 4 and 7, 8 mM $MgCl_2$ + 0.4 mM ouabain. The film was exposed for 3 days at −70 °C.

SDS gel. The larger bands are poorly stained with Coomassie blue, even in these overloaded lanes, and display the diffuse character of the intact glycoprotein. On a higher percentage gel (for example, see Figure 2), the low molecular weight material is resolved as single band of M_r 16 000.[3] The coincidence of the staining and radioactivity patterns for the 40 000- and 31 000-dalton fragments indicates that the tritiated polypeptides are representative of the total population of β molecules. The two large fragments were also observed, by Coomassie blue staining, after papain treatment of unmodified membrane-bound ATPase. In this case, the mobilities were slightly smaller, presumably because of attached sialic acid.

Measurement of the radioactivity in the fragments as a function of papain concentration indicates that most of the label initially present in unproteolyzed β is found in these three species, shown in Table I. The recovery of label is 75–85% except at the highest papain concentration, where it is only 65%. This is probably due to further proteolysis and release of radioactivity into small peptides that generate a diffuse smear on fluorography. The initial rates of appearance of the 16 000- and 40 000-dalton bands are essentially equal and are clearly greater than that of the 31 000-dalton band. The simplest interpretation of these results is that the labeled β chain of M_r 54 000 is hydrolyzed at one site to produce pieces of M_r 16 000 and 40 000. The latter fragment is hydrolyzed at a second site and converted to M_r 31 000. This hypothesis predicts a quantitative result shown in Table I; the ratio of the sum of radioactivities in the M_r 40 000 and 31 000 fragments to the radioactivity in the M_r 16 000 fragment is constant. These data also suggest the existence of at least two carbohydrate chains attached along the β polypeptide. This is consistent with a recent exploration of the biosynthesis of chicken skeletal muscle (Na^+,K^+)-ATPase with a monoclonal antibody which is apparently directed against the β subunit (Fambrough, 1983). Those experiments suggest that three N-linked oligosaccharide chains are present.

[3] It was also possible to effect a change in the mobility of the β chain by using relatively high concentrations of trypsin, chymotrypsin, or V8 protease. Incubation with trypsin generated broad bands of M_r 40 000–45 000 and 20 000–30 000 while treatment with chymotrypsin or V8 protease produced a smear of M_r 45 000–55 000. In experiments with these and other proteases, the fragmentation patterns were more complex than those in the papain digestions, and it was not easy to identify discrete or predominant peptides.

Table I: Distribution of Radioactivity in Papain Fragments[a]

papain (μg)	^3H ($\times 10^{-3}$ cpm)				ratio
	M_r 54 000	M_r 40 000	M_r 31 000	M_r 16 000	
0	11.8	0	0	0	
0.16	7.5	1.1	0.4	1.1	1.4
0.4	4.8	1.8	0.6	1.7	1.4
0.8	2.8	2.6	1.0	2.5	1.4
1.6	1.5	2.7	1.5	2.9	1.4
4.0	0.6	1.8	2.4	2.9	1.4

[a] Labeled ATPase (4 μg) was treated with the indicated amounts of papain and analyzed by SDS gel electrophoresis and fluorography. Portions of the gel were cut out, digested, and counted as described under Materials and Methods. The average values from three separate experiments are presented along with the ratio of the sum of the radioactivities in the M_r 40 000 and 31 000 fragments to the radioactivity in the M_r 16 000 fragment. Background values of radioactivity have been subtracted.

Since the α subunit exhibits conformationally dependent proteolysis with trypsin and chymotrypsin (Chin & Forgac, 1983; Castro & Farley, 1979), it was of interest to examine the papain reactions in the presence of ligands of the (Na^+,K^+)-ATPase. The fragmentation patterns of tritiated ATPase are shown in Figure 2. Hydrolysis of the peptide bonds that generate the M_r 40 000, 31 000, and 16 000 pieces is independent of Na^+ and K^+ (compare lanes 2 and 5 to lanes 3 and 6). Conditions that permit ouabain binding result in a slower rate of hydrolysis and possibly a fourth fragment of M_r 21 000 (lane 7), but these effects are due to a decrease in ionic strength. In a separate experiment, the inclusion of 80 mM choline chloride in the Mg^{2+}/ouabain digestions produced fragmentation patterns identical with those obtained in 80 mM NaCl (lanes 3 and 6). The two sites of papain attack appear to be insensitive to conformations of the (Na^+,K^+)-ATPase.

Although detergents are required for removal of the β subunit from the membrane, the disposition of the polypeptide with respect to the lipid bilayer is not known. In a series of four experiments, the membrane-bound ATPase was digested with papain and then washed at least twice by centrifugation and resuspension. Solutions used for washing were the low ionic strength imidazole buffer, 0.5 M NaCl, and 5 mM EDTA (pH 12). The supernatants and pellets were analyzed by SDS gel electrophoresis for their contents. It was found that none of the washing protocols removed labeled peptides from the membrane pellet, suggesting that all of the papain fragments contain hydrophobic, membrane-embedded regions. An alternative explanation is that the β-subunit fragments are tightly stuck to α-subunit fragments which are embedded in the bilayer.

Studies using hydrophobic photoactivated reagents support the existence of membrane-embedded domains of the β subunit (Giradet et al., 1983; Jorgensen & Brunner, 1983; Montecucco et al., 1981). Giradet et al. (1981) have generated a β-subunit antiserum which still binds to toad kidney microsomes after absorption with intact toad erythrocytes. Since the β chain contains extracellularly exposed carbohydrate, it must span the bilayer if it also has cytoplasmic antigenic determinants. The results presented here indicate that the β chain would have to associate with the membrane in at least two domains to accommodate the two carbohydrate-containing fragments that remain attached to the lipid bilayer. In contrast, a recent report described papain cleavage of duck salt gland (Na^+,K^+)-ATPase β subunit with release of all of the carbohydrate into the supernatant and without appreciable degradation of the α chain or loss of ATPase activity (Freytag, 1983). Although clarification of this discrepancy requires more extensive investigation, one possible explanation is the dif-

Table II: NH_2-Terminal Sequences[a]

M_r 40 000	Ala-(Lys or Pro)-Gly-Lys-Ala-Lys-?-(Leu)-Gly-(Tyr or Lys)-?-Lys
M_r 31 000	Ile-?-Phe-Ile-Pro-(Asn or Lys)-(Thr)-?-Ile
β	Ala-Arg-Gly-Lys-Ala-Lys-Glu-Glu-Gly-Ser-?-Lys-Lys-Phe-Ile-?-Asn-Ser-Glu-Lys-(Pro)-Glu-Phe-Leu-Gly-?-Thr-Asp-?-(Ser)-(Lys)-Phe-Lys
β	Ala-?-Gly-Trp-Ala-Lys-Glu-Glu-Gly

[a] Polypeptides were prepared by SDS gel electrophoresis and sequenced as described under Materials and Methods. The sequences of the papain fragments were obtained from two separate preparations. The upper β-subunit sequence was determined in this work, and the lower sequence (Cantley, 1981) is included for comparison. Unknown residues are represented by a question mark, and possible amino acids are enclosed in parentheses.

ference in sources of the (Na^+,K^+)-ATPase.

Further studies of the path of the β polypeptide with respect to the membrane would be facilitated by a linear alignment of the papain fragments. This approach has already been used for the α subunit (Chin & Forgac, 1983; Castro & Farley, 1979). The two large papain fragments and the intact β subunit were purified by preparative SDS gel electrophoresis, and their NH_2-terminal sequences were determined. The results are given in Table II along with a previous determination of the dog kidney β subunit (Cantley, 1981). The reason for the disagreement in the assignment of residue 4 is unknown. Nevertheless, it is clear that the 40 000-dalton fragment contains the NH_2 terminus of the β subunit and that the 31 000-dalton fragment does not. Therefore, the first papain cleavage removes a 16 000-dalton piece from the COOH terminus of the β subunit, while the second cleavage removes a 9000-dalton piece from the NH_2 terminus.

These data can be incorporated into a model that simply explains all of the results presented in this work. Papain hydrolysis at one site divides the 54 000-dalton β polypeptide into a 40 000-dalton NH_2-terminal peptide and a 16 000-dalton COOH-terminal peptide. Both contain covalently attached carbohydrate and are anchored by at least one membrane-bound stretch of amino acids. The most reasonable interpretation (which is assumed in this model) is that the membrane-bound peptide traverses the lipid bilayer. Nevertheless, it is possible that it enters but does not cross the membrane or that it is tightly associated with integral regions of the α subunit. Higher concentrations of papain result in hydrolysis at a second site that releases a 9000-dalton peptide from the NH_2-terminal end of the 40 000-dalton fragment, leaving a 31 000-dalton peptide which contains the ^3H-labeled galactose residues.[4] The 16 000-dalton peptide need only enter or cross the membrane once; a portion of it is definitely extracellular because of the covalently linked carbohydrate. The membrane-embedded β polypeptide is cleaved at both ends by a water-soluble protease to give the 31 000-dalton fragment. If the two sites are on the same side of the membrane, then the 31 000-dalton peptide must cross the bilayer an even number of times, possibly two. If the two sites are on opposite sides, then it must cross an odd number of times, one or three.

The question of an even or odd number of crossings was addressed in two experiments using a predominantly right-side-out oriented reconstituted (Na^+,K^+)-ATPase (Chin & Forgac, 1984) that had been labeled with tritium prior to solubilization. The reconstituted ^3H-labeled ATPase was treated with varying concentrations of papain in the absence of detergent and analyzed by SDS gel electrophoresis and fluorography. The digestion patterns were identical with those described in Table I; all three labeled fragments were observed. This suggests that both papain sites are accessible from the outside of the vesicle, and, therefore, those peptide bonds are extracellular exposed. As shown in Figure 3, this places the NH_2 terminus outside the cell and the COOH terminus inside with three intervening membrane crossings of the polypeptide chain. This model is presented as a framework for future experiments. It would be interesting to determine the partitioning of the lipid-soluble and digitoxin labels (Giradet et al., 1983; Jorgensen & Brunner, 1983; Montecucco et al., 1981; Farley et al., 1980; Hall & Ruoho, 1980) among the papain fragments.

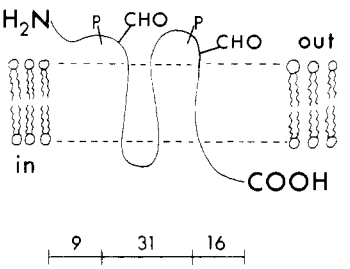

FIGURE 3: Model of the (Na^+,K^+)-ATPase β-subunit structure. The disposition of the β polypeptide with respect to the plasma membrane is shown. The two sites of papain hydrolysis are marked with P. The two sites of carbohydrate attachment are labeled with CHO. The horizontal line indicates the alignment and apparent molecular mass (in kilodaltons) of the papain fragments.

In summary, a method of selectively labeling the (Na^+,K^+)-ATPase β subunit was characterized. It was shown to have no observable effect on the α subunit or on enzyme activity. This method was used to locate and identify proteolytic fragments of the native β chain. Two sites of papain hydrolysis were studied. The fragments were characterized with respect to their apparent molecular weights, their associations with the lipid bilayer, and their NH_2-terminal sequences. On the basis of these results, a model of the linear and three-dimensional structure of the β subunit was proposed.

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Registry No. ATPase, 9000-83-3.

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[4] The 9000-dalton peptide does not appear on fluorography either because it does not contain any radioactivity or because it is further degraded into variously sized pieces that are not well resolved. Although the constant ratio of radioactivities shown in Table I would favor the former explanation, the decrease in recovery of radioactivity would favor the latter.

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Fluorescent Derivatives of Ganglioside G_{M1} Function as Receptors for Cholera Toxin

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ABSTRACT: A fluorescent derivative of ganglioside G_{M1} was prepared by oxidation of the sialic acid residue with sodium periodate and reaction of the resulting aldehyde with Lucifer yellow CH. The biological activity of the fluorescent derivative was compared with that of native G_{M1} using G_{M1}-deficient rat glioma C6 cells. When the cells were exposed to either native or fluorescent G_{M1}, their ability to bind ^{125}I-labeled cholera toxin was increased to a similar extent. This increase in binding was directly proportional to the amount of ganglioside added to the medium. The affinity of the toxin for cells treated with either native or fluorescent G_{M1} also was similar. More importantly, the fluorescent G_{M1} was as effective as native G_{M1} in enhancing the responsiveness of the cells to cholera toxin. Thus, the ganglioside-treated cells exhibited a 9-fold increase in toxin-stimulated cyclic AMP production over cells not exposed to G_{M1}. There was a similar increase in iodotoxin binding and toxin-stimulated cyclic AMP accumulation in cells treated with other G_{M1} derivatives containing rhodaminyl or dinitrophenyl groups. On the basis of these results, it is clear that these modified gangliosides retain the ability to function as receptors for cholera toxin. Consequently, fluorescent gangliosides are likely to be useful as probes for investigating the dynamics and function of these membrane components.

The function of many cell surface components may be related to their mobility in the plane of the membrane (Schlessinger & Elson, 1982). Various methods have been developed to determine the location and movement of plasma membrane constituents. One of the most successful approaches is the introduction of fluorescent probes into such molecules (Taylor et al., 1978; Wilchek et al., 1980; Kreis et al., 1982; Lipsky & Pagano, 1983; Spiegel et al., 1983; Sleight & Pagano, 1984). As a part of our strategy for studying the function of gangliosides, we have prepared fluorescent ganglioside derivatives with an unmodified lipid moiety (Wilchek et al., 1980; Spiegel et al., 1983). These derivatives were used to directly monitor the organization and dynamics of gangliosides in the plasma membrane (Spiegel et al., 1984a,b).

Whenever a biological molecule is chemically modified, a critical question arises as to whether the modification compromises its biological activity. Thus, it seemed important to investigate the effect that covalent attachment of fluorophores to the sialic acid residues of gangliosides has on their activity. In contrast to the vast knowledge available on the chemical structure of gangliosides [see Wiegandt (1982)], little is known about their function. The only well-established function for gangliosides is that of the ganglioside G_{M1}[1] as the receptor for choleragen (Fishman, 1982). When G_{M1}-deficient cells are cultured in medium containing exogenous G_{M1}, the cells take up the G_{M1} and exhibit increased choleragen binding and responsiveness (Moss et al., 1976; Fishman et al., 1980; Fishman, 1980, 1982). In the present study, this procedure was used to assess the biological activity of fluorescent G_{M1} derivatives.

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[1] Abbreviations: G_{M1}, II^{3}NeuAcGgOse$_4$Cer, Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glc-Cer; G_{M3}, II^{3}NeuAcLacCer, NeuAcα2→3Galβ1→4Glc-Cer; DNP, dinitrophenyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, Dulbecco's phosphate-buffered saline (pH 7.4) minus Ca^{2+} and Mg^{2+}; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.