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Three Adenylate Cyclase Phenotypes in S49 Lymphoma Cells Produced by Mutations of One Gene

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SUMMARY

Genetic studies of the S49 mouse lymphoma cell have resulted in isolation and characterization of three mutant cell lines with different phenotypic lesions affecting functions of the guanine nucleotide-binding regulatory component (N protein) of adenylate cyclase: *cyc*$^-$, in which the N protein appears functionally absent; *unc*, in which the N protein's interactions with hormone receptors (R) are impaired; and H21a, in which N appears unable to interact with the catalytic unit (C) of adenylate cyclase. Two-dimensional gel analysis showed that two subunits of the N protein, the 42,000- and 52,000-dalton polypeptides ADP-ribosylated by cholera toxin, have the same size and electrical charge in H21a as in wild-type S49 cells. In addition, the hydrodynamic properties of the detergent-solubilized N protein of H21a membranes were similar to those previously described for the N proteins of wild-type and *unc* S49 cells. We performed genetic complementation analysis of the three mutant phenotypes to determine whether they were produced by lesions affecting the same or different genes. Complementation among the mutant phenotypes was not observed. In particular, the apparently complementary functional defects of H21a and *unc* failed to complement each other in H21a × *unc* somatic hybrids. In these hybrids, N molecules (presumably coded by the H21a genome) were able to regulate the affinity of *beta*-adrenergic receptors for binding isoproterenol, an agonist, whereas other N molecules (presumably coded by the *unc* genome) mediated stimulation of C by guanine nucleotides, NaF, and cholera toxin. As in both the H21a and *unc* parental cell lines, however, R-C coupling was deficient in the hybrid cells, in which adenylate cyclase remained unresponsive to stimulation by hormones. These results indicate that the *cyc*$^-$, *unc*, and H21a phenotypes are caused by different mutations in a common gene.

INTRODUCTION

Current models of hormone-sensitive adenylate cyclase (1–5) assign a central role to its guanine nucleotide-binding regulatory component (N[1]), which functionally couples hormone receptors (R) to stimulation of catalytic adenylate cyclase (C). The N protein is thought to shuttle between R and C in a cycle that involves binding and hydrolysis of GTP, followed by release of GDP. Biochemical evidence suggests that the N-GTP complex can bind to and activate C (6) and that, in the absence of guanine nucleotides, N can bind to the hormone-receptor (HR) complex (7). Both processes appear to be essential for transduction of the external hormonal signal into synthesis of intracellular cyclic AMP.

The N protein purified from rabbit liver contains three polypeptide subunits with molecular weights of 52,000, 45,000, and 35,000 (8, 9). To understand the molecular details of R-C coupling by the N protein, it will be necessary to define the roles of individual subunits of N. Cholera toxin catalyzes ADP ribosylation of the two larger subunits (10), but not of the 35,000-dalton polypeptide (8, 9). Photoaffinity labeling experiments in an avian erythrocyte system (11) suggest that GTP binds to a site on a subunit that is also a substrate for cholera toxin. Exposure of the N protein to NaF or guanine nucleotide analogues reduces the sedimentation velocity of N activity (9, 12) or radiolabeled cholera toxin substrate (6) in sucrose gradients, raising the possibility that N subunits dissociate when the protein is activated. Further biochemical investigation is required to deter-

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[1] The abbreviations used are: N, guanine nucleotide-binding regulatory component of adenylate cyclase; R, hormone receptor; C, catalytic unit of adenylate cyclase; *cyc*$^-$, mutant S49 cell in which N is functionally deficient; *unc*, mutant S49 cell in which N appears uncoupled from R; H21a, mutant S49 cell in which N appears uncoupled from C; HPRT$^-$, deficiency of hypoxanthine-guanosine-phosphoribosyltransferase; TK$^-$, deficiency of thymidine kinase; HAT medium, combination of hypoxanthine, aminopterin, and thymidine; PGE_1, prostaglandin E_1.

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mine which of the subunits are primarily responsible for interactions with R or C, and to define the function of the 35,000-dalton subunit.

A different approach, utilizing somatic genetics, may also help to unravel the complex functions of the N protein. Three mutant cell lines derived from the S49 mouse lymphoma cell exhibit distinct phenotypic alterations of adenylate cyclase, caused by different functional defects of the N protein: N protein activity appears completely deficient in the S49 *cyc*$^-$ mutant (1–3, 13) in which adenylate cyclase is unresponsive to stimulation by hormones, guanine nucleotides, NaF, and cholera toxin (14). Adenylate cyclase in S49 *unc* cells (15) is specifically unresponsive to stimulation by hormones, and interactions of the N protein with R in these cells are specifically impaired or "uncoupled." Recently we described a third phenotype, in cells of a clone termed H21a (16, 17), in which N is able to interact with R but not with C; the affinity of *beta*-adrenergic R for binding hormonal agonists is preserved in H21a, but adenylate cyclase in these cells fails to respond to the same array of stimuli that are also ineffective in *cyc*$^-$. Two-dimensional gel analysis showed that the 52,000- and 42,000-dalton polypeptide N subunits ADP-ribosylated by cholera toxin had the same size and electrical charge in H21a as in wild-type S49 (17), and confirmed the previously described (18) acidic charge shift of these subunits in *unc*.[2]

Accordingly, we ask in the present report whether the apparently reciprocal functional deficiencies of N in *unc* and H21a cells are due to mutations affecting different subunits of the protein. A biochemical approach found no alteration in the hydrodynamic properties of the N oligomer of H21a, as compared with wild-type. Genetic complementation analysis of somatic hybrid cells was more successful. Hormones did not stimulate cyclic AMP synthesis in H21a × *unc* hybrid cells, although these cells expressed the defective gene products of both parental cells. Failure of the two phenotypes to complement each other's defects indicates that the *unc* and H21a defects are caused by mutations in a single gene. Additional experiments indicated that the *cyc*$^-$ phenotype is caused by a mutation in the same gene, which is probably the structural gene that codes for both the cholera toxin substrate polypeptides of the N protein.

MATERIALS AND METHODS

Cells. S49 cells were propagated as described (14). Table 1 lists the cell lines used. The parental cell lines were derived from wild-type S49 [clone 24.3.2], *unc* [provided by Dr. A. G. Gilman (15)], *cyc*$^-$ (14, 20), and H21a (16). Hybridization markers in the parental lines were selected by proliferation of cells in agar containing 6-thioguanine (to isolate HPRT$^-$ cells) or cells resistant to killing by bromodeoxyuridine (to isolate TK$^-$ cells) (20). These parental cell lines were unable to proliferate in HAT medium (20).

[2] The 42,000-dalton peptide radiolabeled in this (10, 17, 19) and other laboratories (6, 7) is identical with the 45,000-dalton peptide reported by Gilman and his collaborators (8, 9, 18); the 3,000-dalton discrepancy presumably is due to differences in techniques or protein standards.

TABLE 1
Cell lines

Parental lines		Hybrid lines	
Phenotype	Clone	Cross	Clone
Wild-type	24.3.2	H21a × wild-type (B1.1.6. × U36)	B16.3.18
Wild-type, TK$^-$	U36	H21a × *unc* (B1.1.6 × B5.8.1)	B11.2.37
unc, TK$^-$	B5.8.1.	H21a × *cyc*$^-$ (B1.1.6 × M3.B1)	B10.6.21
unc, HPRT$^-$	M2.1	*unc* × *cyc*$^-$ (M2.1 × M3.B1)	B13.4.5
H21a, HPRT$^-$	B1.1.6		
cyc$^-$, TK$^-$	M3.B1		

Hybrids. Hybrids were formed by treating a mixture of two parental cell lines with polyethylene glycol (20). Hybrid cell lines were then selected by cloning in soft agar containing HAT medium (20), with hybridization efficiencies of between 6 and $10/10^5$ parental cells. These cells were presumed to be hybrids on the basis of their ability to proliferate in HAT medium, a doubling in apparent cell volume (assessed in comparison to beads of standard size in a Coulter Channelyzer) and a doubling of the modal number of chromosomes (79–81 chromosomes in each of the hybrid lines versus 40–41 chromosomes in the pseudodiploid parental S49 lines). In addition, control hybridization experiments using parental lines with a single HAT marker (HPRT$^-$ or TK$^-$) produced no clones capable of proliferating in agar-containing HAT medium.

Membranes. Partially purified plasma membranes were prepared by a modification (10) of the method of Ross *et al.* (21).

Functions of N in cells and membranes. Accumulation of cyclic AMP in logarithmically growing cells and adenylate cyclase activities in membrane preparations were measured as described (16). The ability of *beta*-adrenergic receptors to bind the agonist isoproterenol with high affinity was assessed in competition binding experiments using the radiolabeled *beta*-antagonist, [^{125}I]iodohydroxybenzylpindolol (16).

Hydrodynamic properties of N. Wild-type or H21a membranes (1 mg/ml) were incubated with 25 μM [^{32}P] NAD (10 Ci/mmole) for 30 min in the presence of activated cholera toxin (50 μg/ml) (22). The membranes were washed by centrifugation, and N was solubilized in 0.7% (w/v) lubrol PX. Appropriate aliquots of the 100,000 × *g* supernatant fractions of the Lubrol-soluble extracts were chromatographed on Sepharose 6B or subjected to centrifugation through 5–20% sucrose gradients (19). N activity (of wild-type extracts) in column or gradient fractions was measured by its ability to complement the adenylate cyclase defect of *cyc*$^-$ membranes *in vitro* (19). For both wild-type and H21a extracts, aliquots of each column or gradient fraction were treated with 2% sodium dodecyl sulfate and 5% β-mercaptoethanol and subjected to electrophoresis on 10% polyacrylamide gels (10). After autoradiography of the gels, slices corresponding to the 42,000-dalton radiolabeled band were cut from the gels and counted in a scintillation spectrometer (19). Protein standards in the gel filtration and sucrose gradient determinations included β-galactosidase, fumarase, lactate dehydrogenase, malate dehydrogenase, and cytochrome *c*. The hydrodynamic properties of these proteins have been summarized (12).

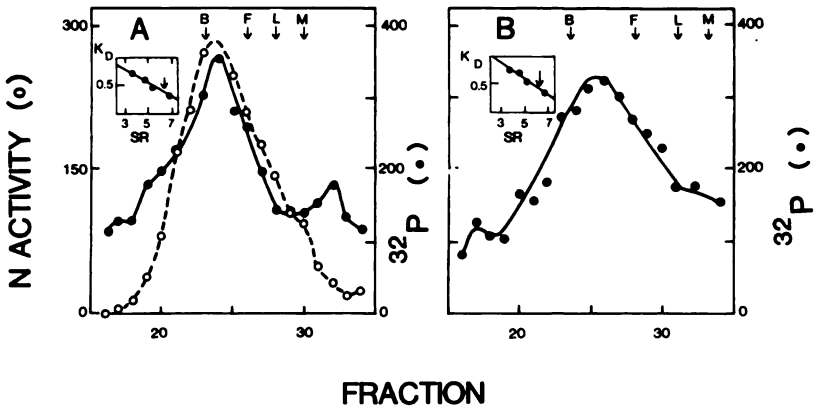

FIG. 1. *Sepharose 6B chromatography of Lubrol-soluble extracts of wild-type (A) and H21a (B) membranes*

Membranes were treated with cholera toxin and [^{32}P]NAD and solubilized in Lubrol, as described under Materials and Methods. The 100,000 × *g* supernatant fraction of each Lubrol extract (0.125 ml) was chromatographed on a Sepharose 6B column (0.5 × 19 cm) and fractions of 0.125 ml were collected. Values for N activity (○), assayed by *in vitro* complementation of *cyc*$^-$ membranes (19), are picomoles of cyclic AMP per 40 minute per milliliter of column eluate. ^{32}P values (●) represent counts per minute incorporated into the 42,000-dalton polypeptide per column fraction, assayed as described under Materials and Methods. *Arrows* represent positions of marker proteins (*B* = β-galactosidase; *F* = fumarase, *L* = lactate dehydrogenase; *M* = malate dehydrogenase). *Insets* in A and B show K_D of marker proteins (ordinate) plotted versus their Stokes radii (in nanometers); *arrows* in the *insets* indicate the position of N activity and/or the radiolabeled 42,000-dalton polypeptide.

RESULTS

Gel filtration and sucrose gradient sedimentation of N in wild-type and H21a. N activity in Lubrol extracts of wild-type membranes migrated on Sepharose 6B (Fig. 1A) at a position corresponding to a Stokes radius of 6.1 ± 0.15 nm ($n = 3$), which was similar to values previously determined for N activity from S49 cells (12) and from human erythrocytes (19). The ^{32}P radiolabel in the 42,000-dalton peptide subunit of wild-type N comigrated with wild-type N activity on Sepharose 6B (Fig. 1A). Because N in H21a extracts does not complement *cyc*$^-$ *in vitro* (16), it was possible only to determine the Stokes radius of the ^{32}P radiolabel in the 42,000-dalton substrate of cholera toxin (Fig. 1B).[3] This peptide in H21a extracts migrated at a position corresponding to a Stokes radius of 6.2 ± 0.14 nm ($n = 3$), which did not differ significantly from wild-type.

Sucrose gradients (Fig. 2) gave more complex results. N activity in wild-type extracts (Fig. 2A) sedimented at 5.0 ± 0.1 S (mean ± SEM; $n = 3$), similar to the values previously determined for N from wild-type S49 (12) and human erythrocytes (19). The ^{32}P radiolabel in the 42,000-dalton band, however, consistently migrated in a broad bifid peak; the more rapidly sedimenting component of this peak (which usually contained less radioactivity) comigrated with N activity at 5.1 ± 0.1 S. The nature of the less rapidly migrating component is unclear, although it could represent denatured or inactive protein; inhibitors of protease activity did not affect the presence or size of this component.[4] H21a extracts (Fig. 2B) also consistently showed a broad, bifid peak of radiolabel in the 42,000-dalton band. We assume that the more rapidly migrating component of this peak corresponds to undenatured N protein in H21a. Relative to the marker proteins, this component had a sedimentation coefficient of 4.7 ± 0.1 S ($n = 3$), slightly smaller than that of wild-type in the same experiments. The slowly migrating component of the H21a peak appeared similar to that observed in wild-type.

In summary, these experiments provided no evidence that the N protein of H21a differs in size from that of wild-type. Assuming that both proteins have a partial specific volume similar to that of soluble proteins, their molecular weights can be calculated from experimentally determined values for Stokes radius and $s_{20,w}$.[5] The calculated molecular weight of the wild-type protein (134,000 ± 4,200) is not statistially different from that of the H21a protein (128,000 ± 4,400) ($p > 0.20$).

Genetic complementation analysis: cyclic AMP synthesis in response to hormones and other effectors. Measurements of cyclic AMP accumulation in intact cells (Fig. 3) and adenylate cyclase activity in membranes (Fig. 4) showed the characteristic phenotypic defects of *unc* (15), *cyc*$^-$ (14), and H21a (16) in the mutant cell lines used as parents for the hybridization experiments. Cyclic AMP synthesis in *unc* cells and membranes responded to stimulation by cholera toxin, guanylyl-5′-imidodiphosphate, and NaF, but was unresponsive to isoproterenol and PGE_1. Cyclic AMP synthesis in *cyc*$^-$ and H21a responded to none of these effectors. Mn^{2+} ion, which is thought to stimulate the catalytic unit directly (20, 23), stimulated adenylate cyclase activity in all the mutants, as well as in wild-type (Fig. 4).

Genetic complementation analysis tested the hypothesis that defective responsiveness of adenylate cyclase in

[3] Radioactivity in the 52,000-dalton polypeptide was not measured; visual inspection of autoradiograms indicated that the larger subunit comigrated with the 42,000-dalton subunit.

[4] The bifid peak of radioactivity in sucrose gradients was consistently observed with different batches of wild-type and H21a membranes, but never in experiments with erythrocyte membranes. Phenylmethanesulfonate (170 μg/ml) and aprotinin (100 units/ml) had no effect on the shape of these peaks.

[5] The N proteins of wild-type S49 (12) and human erythrocytes (19) bind little Lubrol, so that the partial specific volume of the protein in these gradients was probably close to 0.73 ml/g (12, 19). On the basis of this assumption, calculation of molecular weights from Stokes radius and sedimentation velocity was performed exactly as described (19).

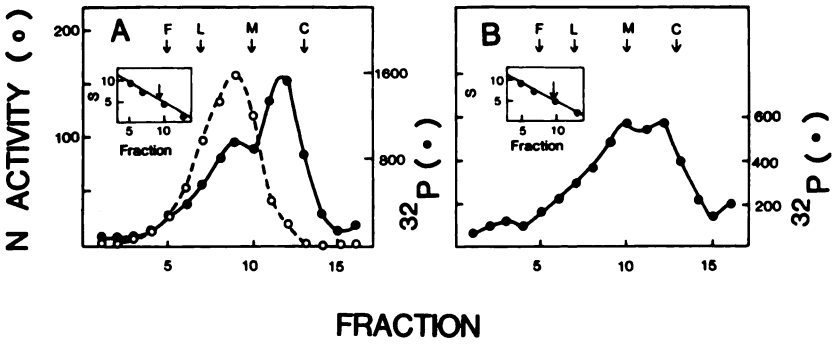

FIG. 2. *Sucrose gradient sedimentation profiles of Lubrol extracts from wild-type (A) and H21a (B) membranes*

Membranes were treated with [^{32}P]NAD and solubilized with Lubrol, as described under Materials and Methods. The 100,000 × *g* supernatant fraction of each Lubrol extract (0.25 ml) was applied to a 5-ml 5–20% sucrose gradient and centrifuged at 40,000 rpm in a Beckman SW 50.1 rotor for 15 hr, as described (19). N activity (○, picomoles of cyclic AMP per 40 minute per milliliter of gradient fraction) and ^{32}P incorporation into the 42,000-dalton polypeptide (●, counts per minute per fraction) were assayed as described under Materials and Methods. *Arrows* represent positions of cytochrome *c* (*C*) and other marker proteins, abbreviated as described in the legend to Fig. 1. *Insets* in A and B show $s_{20,w}$ values for the marker proteins (ordinate) plotted versus column fraction. *Arrows* in the *insets* indicate the position of N activity and/or the more rapidly moving shoulder of the peak of ^{32}P-radiolabel in the 42,000-dalton peptide. The broad, bifid peak of radiolabel was consistently observed in extracts of different membrane preparations of both wild-type and H21a.

the three mutant phenotypes is caused by mutations in different genes. Thus, the apparently reciprocal defects of H21a and *unc* cells might be due to defects in different peptide gene products, of which one (altered in H21a) is required for N to stimulate C whereas the other (altered in *unc*) is required for interactions of N with HR; if so, the H21a × *unc* somatic hybrid should be able to synthesize cyclic AMP in response to hormones, because each parental genome would supply the gene product that is lacking or defective in the other parent. This result was not found. Instead, H21a × *unc* hybrid cells accumulated cyclic AMP in response to cholera toxin, but not isoproterenol or PGE_1 (Fig. 3), and adenylate cyclase in membranes of the hybrid was phenotypically *unc*, rather than wild-type (Fig. 4). Failure of the two mutant phenotypes to complement each other's defects in the hybrid makes it highly likely that both are caused by mutations in the same gene.

Similarly, both the H21a × *cyc*$^-$ and the *unc* × *cyc*$^-$ hybrids showed failure of genetic complementation, indicating that the three parental cells bear mutations in a common gene. The H21a × *cyc*$^-$ hybrid exhibited the H21a phenotype, and the *unc* × *cyc*$^-$ hybrid exhibited the *unc* phenotype (Figs. 3 and 4). The latter observation confirms a result reported previously (20), in which a different *unc* × *cyc*$^-$ hybrid was examined.

A necessary condition for genetic complementation analysis is that the mutant phenotypes to be tested must behave as recessive with respect to the wild-type phenotype; i.e., the mutant phenotypes must not suppress expression of normally functioning gene products.[6] Previous experiments (20) showed that both the *unc* and the *cyc*$^-$ phenotypes are recessive to wild-type, in that adenylate cyclase in wild-type × *unc* and wild-type × *cyc*$^-$ hybrids responds to all of the effectors that stimulate cyclic AMP synthesis in the wild-type parent. Similarly, the wild-type × H21a hybrid expresses a wild-type phenotype (Figs. 3 and 4).

Cholera toxin substrates. The 42,000- and 52,000-dalton polypeptides radiolabeled in the presence of cholera toxin and [^{32}P]NAD were detectable in wild-type, *unc*, and H21a membranes, but not in *cyc*$^-$ (Fig. 5), as previously described (10, 17). These two subunits of the N protein were also present in membranes of all the hybrid cell lines tested (Fig. 5). In particular, note that hybrids

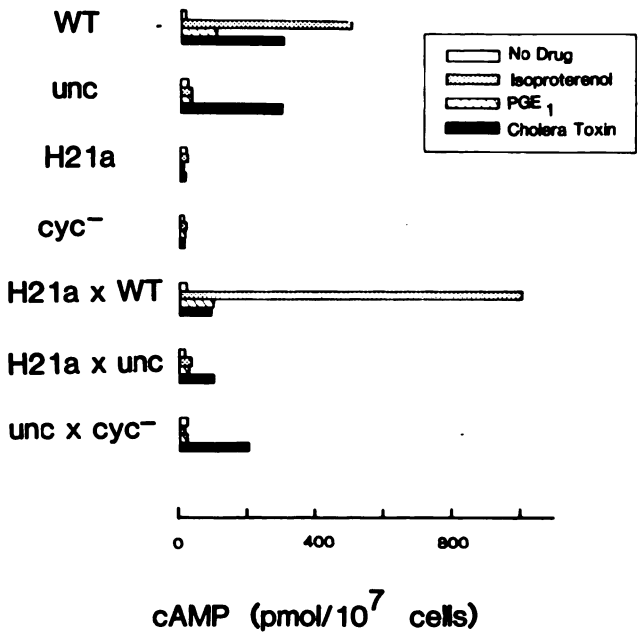

FIG. 3. *Cyclic AMP accumulation by S49 wild-type (WT), mutant, and hybrid cells in response to various effectors*

Cells in the logarithmic phase of growth were exposed to drugs in the presence of 10 μM 3-isobutyl-1-methylxanthine, an inhibitor of cyclic nucleotide phosphodiesterase. For each cell type the stimulators (from *top* to *bottom*) included: no drug, isoproterenol (1 μM), PGE_1 (1 μM), or cholera toxin (500 ng/ml). Cellular cyclic AMP was assayed as described (16) after drug treatment for 15 min (for isoproterenol and PGE_1) or 90 min (for cholera toxin).

[6] If the mutant phenotype were dominant in a cross with wild-type, it might also suppress expression of normal genes in a cross with another mutant. In this case, complementation would not occur, even if the parental cells had mutations in different genes.

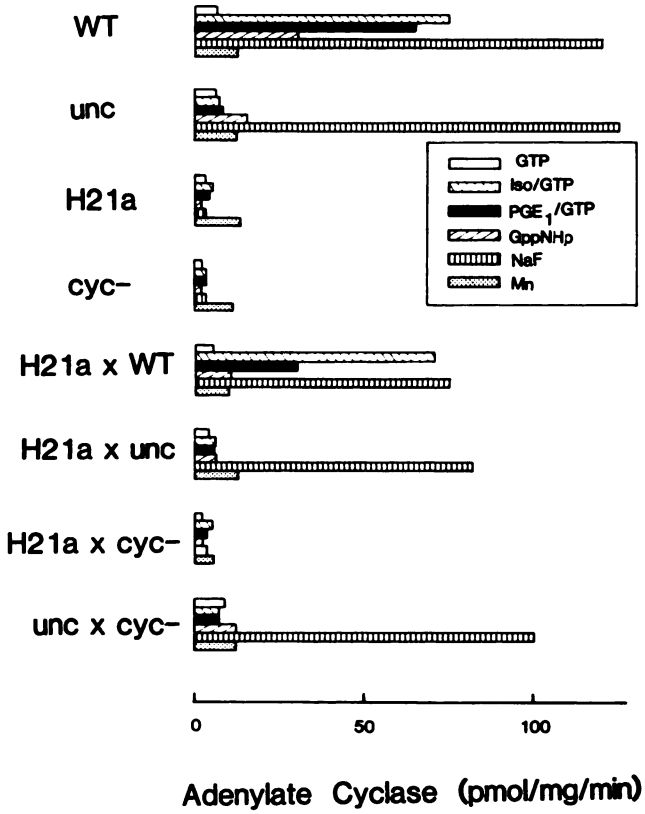

FIG. 4. *Adenylate cyclase activity in membranes of wild-type (WT), mutant, and hybrid cells stimulated by various effectors*

For each membrane type, stimulators (*top* to *bottom*) included: 100 μM GTP, GTP plus 100 μM isoproterenol, GTP plus 10 μM PGE_1, 100 μM guanylyl-5′-imidodiphosphate, 10 mM NaF, or 10 mM $MnCl_2$.

constructed between *cyc*⁻ and either *unc* or H21a express both toxin substrate peptides; presumably these peptides are coded by the *unc* or H21a genome in these hybrids, rather than by the *cyc*⁻ genome (see below).

Agonist binding to beta-adrenergic receptors. In membranes of many cell types, including wild-type S49 (21), guanine nucleotides reduce the affinity of *beta*-adrenergic receptors for binding agonists such as isoproterenol. Biochemical and genetic evidence (1, 2, 7, 24) suggests that the high affinity of receptors for agonists requires an interaction between R and N, and that guanine nucleotides disrupt this interaction. Thus, membranes of mutant cells in which N is functionally absent (*cyc*⁻) or uncoupled from R (*unc*) contain receptors that bind isoproterenol with low affinity, even in the absence of guanine nucleotides (15, 21); receptors in such membranes acquire the capacity to bind agonists with high affinity when exogenous N molecules are added *in vitro* (24). In H21a membranes, as in wild-type, *beta*-adrenergic receptors bind isoproterenol with high affinity in the absence of guanine nucleotides, and with low affinity when guanine nucleotides are present (17). This observation suggested that N in H21a is able to interact normally with R, although it is apparently uncoupled from C.

Accordingly, we sought to determine whether H21a × *unc* and H21a × *cyc*⁻ hybrids contained N activities that could allow *beta*-adrenergic receptors to bind isoproterenol with high affinity. In membranes of both hybrids, isoproterenol competed with high affinity for receptors, and GTP reduced this affinity (Fig. 6), just as in wild-type and H21a membranes (17). These results constitute important controls for interpreting the phenotypic patterns of cyclic AMP accumulation and adenylate cyclase in the hybrids (Figs. 3 and 4), because they indicate that the N protein coded by the H21a genome is expressed in both hybrids. Because the H21a × *unc* hybrid expressed mutant genes derived from both parents, failure of complementation in the hybrid (i.e., unresponsiveness of adenylate cyclase to stimulation by hormones) makes it highly likely that both parental mutations involve the same gene. The failure of the H21a gene product to complement the *cyc*⁻ defect suggests that these two parental cells bear mutations in a common gene as well.

Finally, the phenotype of the *unc* × *cyc*⁻ hybrid confirms the other two results, indicating that all three mutations involve a common gene. The adenylate cyclase of *unc* × *cyc*⁻ hybrids showed no response to stimulation by isoproterenol or PGE_1 (Figs. 3 and 4); in addition, *beta*-adrenergic receptors in these hybrid cells, like the receptors of the two parental cell lines, bound isoproterenol with low affinity, whether GTP was present or not (Fig. 6).

DISCUSSION

We studied three S49 mutants that exhibit different phenotypic defects in functions of the N protein: uncoupling from R in *unc*, uncoupling from C in H21a, and loss of interactions with both R and C, combined with apparent disappearance of cholera toxin substrates, in *cyc*⁻. Recent evidence that the N protein is an oligomer composed of dissimilar polypeptide subunits (8, 9) raised the

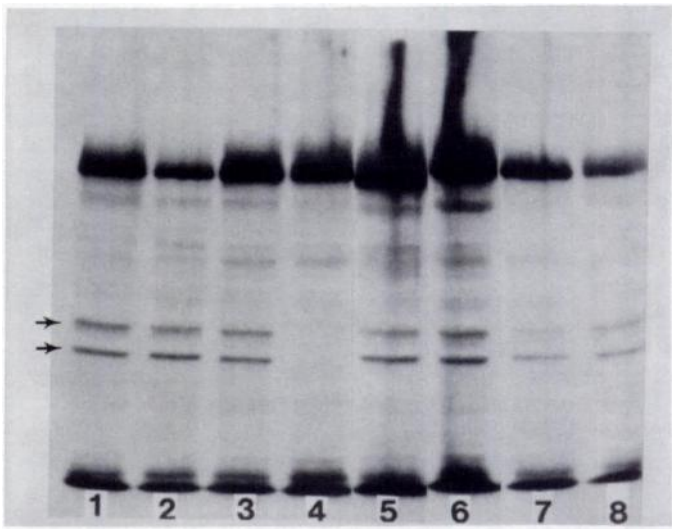

FIG. 5. *Autoradiograms of S49 cell membranes incubated with [^{32}P]NAD and cholera toxin and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis*

Each numbered lane contained extract from a different cell type, as follows: *1*, wild-type; *2*, *unc*; *3*, H21a; *4*, *cyc*⁻; *5*, H21a × *cyc*⁻; *6*, H21a × *unc*; *7*, *unc* × *cyc*⁻; *8*, H21a × wild-type. Bands corresponding to the 42,000- and 52,000-dalton subunits of N are indicated by *arrows*. These bands were not labeled if cholera toxin was omitted from the labeling mixture (data not shown).

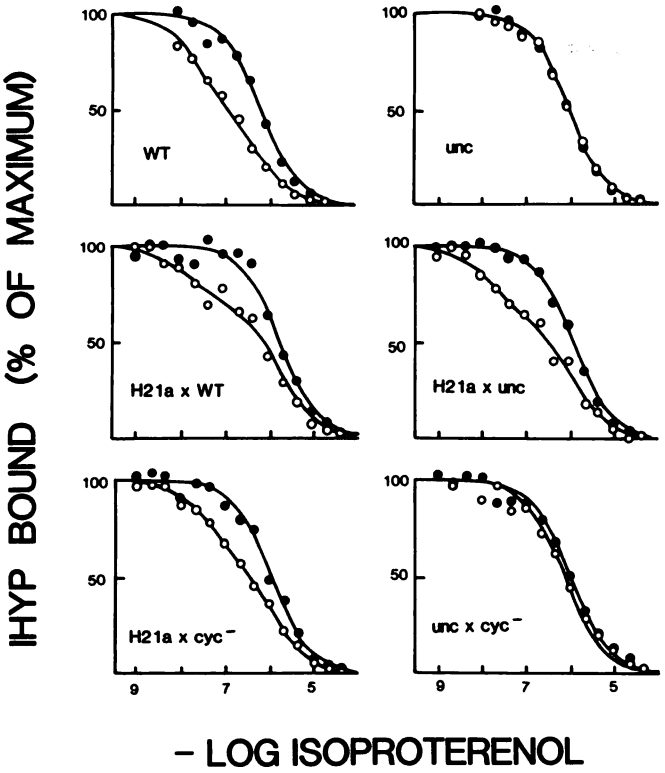

FIG. 6. *Competition by isoproterenol for [^{125}I]iodohydroxybenzylpindolol (IHYP) binding sites in membranes of wild-type (WT), mutant, and hybrid cells*

Various concentrations of isoproterenol (abscissa) were incubated with an approximate K_D concentration (72 pM) of [^{125}I]IHYP and membranes in the presence (●) or absence (○) of 300 μM GTP, and binding was measured exactly as described (17). Maximal binding (ordinate) represents the binding of radioligand in the absence of isoproterenol. Specific binding capacity for [^{125}I]IHYP, determined as described (17), ranged from 150 to 225 fmoles/mg of protein in these membrane preparations. Competition binding curves for each type of membrane were performed at least three times with more than one membrane preparation; results in each case were similar to those shown.

possibility that these different phenotypes might be caused by mutations of different genes. Instead, the present work indicates that all three phenotypes are caused by mutations in a single gene.

Absence of complementation between the hybrid phenotypes coincides with the parallel failure of biochemical complementation, in experiments that involved adding N from *unc* to *cyc*$^-$ membranes (24) and adding N from H21a to either *unc* or *cyc*$^-$ membranes (17). None of these biochemical complementation experiments resulted in assembly of adenylate cyclase responsive to hormones. In comparison to the biochemical experiments, the hybrid results constitute more persuasive evidence that all three cyclase phenotypes are caused by mutations of a single gene, for two reasons:

1. In the biochemical experiments with H21a, it was not definitively established that the N protein from H21a became incorporated into *unc* or *cyc*$^-$ membranes. In contrast, the hybrid phenotypes show that N from H21a was expressed in membranes of hybrid crosses with either *unc* or *cyc*$^-$, and hormone-sensitive adenylate cyclase was not reconstituted.

2. The hybrid phenotypes rule out an alternative explanation of the biochemical complementation results, according to which two of the mutants could bear lesions in different genes. According to this view (18), *unc* might present functional loss of a gene product whose action (e.g., covalent modification of N subunits) is necessary for receptor-cyclase coupling; *cyc*$^-$ membranes might not be able to complement this defect *in vitro*, either because *cyc*$^-$ has two lesions or because the gene product that is defective in *unc* is not a membrane protein. Such an explanation is not consistent with the failure of H21a to complement either the *cyc*$^-$ or the *unc* defect in hybrids, or with the failure of *unc* and *cyc*$^-$ to complement one another in hybrids.

We propose that all three mutations are in a structural gene that codes for both the 42,000 and 52,000-dalton subunits of the N protein. This interpretation is consistent with the coordinate loss of detectable cholera toxin-catalyzed ADP ribosylation of both these subunits in *cyc*$^-$ (10), with the similar proteolytic peptide maps of the two polypeptides in wild-type and other cells (25), and with the observation (18) that the isoelectric points of both polypeptides in *unc* are more acidic than those in wild-type, by approximately 1 unit of electrical charge. According to this interpretation, the 35,000-dalton subunit of N, which is not a substrate for ADP ribosylation, might be present in normal amounts (i.e., comparable to wild-type) in membranes of all three mutants. Northup, Gilman, and their co-workers have recently found equivalent amounts of activity attributable to the 35,000-dalton subunit in membranes of both *cyc*$^-$ and H21a cells.[7]

If both the 52,000- and 42,000-dalton polypeptides are coded by the same structural gene, it is likely that the smaller polypeptide is produced by proteolytic cleavage of the larger polypeptide. It is not clear whether this conversion would have funtional significance. Sternweis *et al.* (9) recently reported that N protein preparations (purified from rabbit liver) entirely lacking the 52,000-dalton subunit could mediate cyclase stimulation by hormones when added to *cyc*$^-$ membranes; N protein preparations relatively enriched in 52,000-dalton subunits, however, coupled hormone receptors more efficiently to stimulation of cyclic AMP synthesis. The 42,000-dalton polypeptide is probably not essential for mediating hormonal response, because hormone-sensitive adenylate cyclases have been observed in cells that appear to contain only the larger polypeptide (22).

Our assignment of both the *unc* and H21a mutations to the gene that codes for the cholera toxin-substrate polypeptides implies that these polypeptides contain two functional domains. One of these, altered in *unc*, is involved in interaction with R, whereas the other, altered in H21a, is involved in the protein's interaction with C. Further biochemical investigation is required to determine how these postulated domains are structurally related to the guanine nucleotide-binding site, the site that is ADP-ribosylated by cholera toxin, and the portion of these polypeptides that binds to the 35,000-dalton subunit of the protein.

Finally, the hydrodynamic data indicate that func-

[7] N. Northup, personal communication.

tional lesions in these hypothetical domains are not associated with loss of mass in the mutant N proteins. Apparent sizes of the N proteins in H21a (Figs. 1 and 2) and *unc* (12) do not differ appreciably from that of N in wild-type.

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