Reconstitution of Catecholamine-sensitive Adenylate Cyclase

RECONSTITUTION OF THE UNCOUPLED VARIANT OF THE S49 LYMPHOMA CELL*

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The uncoupled (UNC) variant of the S49 lymphoma possesses receptors and other known regulatory components necessary for hormone-stimulated adenylate cyclase activity but fails to respond to β-adrenergic agonists or to prostaglandin E1. A procedure is described for the reconstitution of responses to hormones in purified plasma membranes of these cells. This technique utilizes cholate extracts prepared from membranes of wild type S49 cells or of membranes of other cell lines that lack either β-adrenergic receptors or the catalytic subunit of adenylate cyclase. The procedure also restores adenylate cyclase activity to membranes of the adenylate cyclase-deficient (cyc-) S49 cell variant.

Hormone responses of reconstituted UNC and cyc- membranes resemble those of wild type S49 cell membranes in their dependence on agonist concentration for both enzyme activation and binding to β-adrenergic receptors. Effects of guanine nucleotides on the binding of agonists, which are lost in UNC and cyc- membranes, are also restored in the reconstituted preparations.

The factors in the cholate extract that are required for reconstitution of the two variant membranes are similarly labile to heating at 37°C and to treatment with N-ethylmaleimide but are differentially sensitive to treatment with trypsin. A protein is thus required for the reconstitution of UNC membranes. This protein appears to be closely linked to and, at the present time, is not distinguishable from the protein necessary for the restoration of adenylate cyclase activity in cyc-.

Analysis of the components that comprise a hormone-sensitive adenylate cyclase is at present limited to observation of the various activities that are expressed by the enzyme system. Resolution and reconstitution of some of these activities have led to the identification of at least three distinct components of adenylate cyclase systems that are stimulated by β-adrenergic agonists. A catalytic entity has been clearly separated from the catalyst itself (C') and a regulatory protein (G/F); a complementary hepatoma clone, HC-1, which is also devoid of adenylate cyclase activity, retains the regulatory protein but is deficient in the activity of the catalyst itself (4).

The existence of the uncoupled variant of the S49 lymphoma suggests a fourth possible candidate for constituency in hormone-sensitive adenylate cyclase (8). The UNC variant possesses an active adenylate cyclase that is responsive to stimulation by both guanine nucleotides and fluoride; in addition, these cells retain an apparently normal complement of β-adrenergic receptors. By current definition, therefore, these cells possess the identified protein components of the enzyme system. However, neither β-adrenergic agonists nor prostaglandins are capable of stimulating adenylate cyclase activity in either intact UNC cells or in plasma membranes derived therefrom.

To facilitate identification of the defect in the UNC cell membrane, we have developed a method to reconstitute its hormone-sensitive adenylate cyclase activity; this procedure utilizes detergent extracts prepared from membranes of both complementary and fully competent cells. This report provides a description of this method and of some of the characteristics of the factor(s) required for this reconstitution.

EXPERIMENTAL PROCEDURES

The cell lines used in this study have been described previously, and their phenotypic properties are summarized in Table I. S49 clones (wild type and variants) were grown in suspension culture at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum. R82 cells were also grown in suspension, but in Spinner-modified Eagle's medium containing 5% heat-inactivated horse serum. Cells were harvested, homogenized, and fractionated as described previously (5, 9). The abbreviations used are: Gpp(NH)p, guanylyl-5'-yl imidodiphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; cyc-, an S49 lymphoma variant clone phenotypically deficient in adenylate cyclase activity; UNC, an S49 lymphoma variant clone that has NaF- and Gpp(NH)p-stimulated adenylate cyclase and β-adrenergic receptors but in which the effect of hormones on the enzyme has been lost; C and G/F, two resolved components of adenylate cyclase. The properties of these components are described in the text.
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Table I

| Cell line | Source | Pheno-typea | Reference |
|-----------|--------|-------------|-----------|
| S49-wild type | Mouse lymphoma | R_{-}^{+} R_{-}^{+} G/F^{-} C^{-} | (9) |
| S49-cyc | Mouse lymphoma | R_{-}^{+} R_{-}^{+} UNC G/F^{-} C^{-} | (3, 4, 7, 9 11) |
| S49-UNC | Mouse lymphoma | R_{-}^{+} R_{-}^{+} UNC G/F^{-} C^{-} | (8) |
| B82 | Mouse fibroblast | R_{-}^{+} R_{-}^{+} G/F^{-} C^{-} | (12, 13) |
| HC-I | Mouse hepatoma | R_{-}^{+} R_{-}^{+} G/F^{-} C^{-} | (4, 10) |

a R\textsuperscript{-}, G/F\textsuperscript{-}, and C\textsuperscript{-} denote, respectively, the presence of \(\beta\)-adrenergic (\(\beta\)) and prostaglandin (\(\pi\)) receptors, a regulatory component required for adenylate cyclase activity, and the catalytic component of adenylate cyclase. A cell line is designated UNC if it displays an uncoupled state between the receptors and adenylate cyclase that is not explicable by a deficiency of a known component of the system. The presence of prostaglandin receptors in cyc\textsuperscript{-} and UNC is not definitely established but strongly indicated in this study.

RESULTS

Extraction of wild type S49 membranes with sodium cholate, as described under "Experimental Procedures," results in a 70% reduction in fluoride-stimulable adenylate cyclase activity and a total loss of the response to \(\beta\)-adrenergic agonists and prostaglandin E\textsubscript{1}. Adenylate cyclase activity can be found in the detergent extract, but it is extremely labile during assay at 30°C unless the detergent is diluted sufficiently. This lability is more clearly defined by a loss of activity that occurs with a half-time of 3 min when the extract is incubated at 25°C; it is due to selective inactivation of the catalytic subunit, C, as will be seen from the reconstitutions to be described below. We have used a room temperature incubation as a convenient means to inactivate C and thus to eliminate the possible contribution of soluble C activity to the reconstitution of UNC and cyc\textsuperscript{-} membranes, both of which contain this protein.

Reconstitution of Membranes from UNC and cyc\textsuperscript{-} S49 Cells—Cholate extracts of wild type S49 cells are able to restore a stable hormone response to membranes from the UNC variant. A simple titration, in which increasing amounts of cholate extract were combined with a fixed amount of UNC membranes, is shown in Fig. 1A; stimulation of the enzyme by isoproterenol is clearly restored. However, both the basal (GTP) and fluoride-stimulated activities of the UNC membranes are also increased following addition of the extract, until an optimum is reached. The decline of all activities that is observed with larger amounts of extract is, apparently, a negative response to further elevation of the detergent concentration. The sole addition of cholate to UNC membranes in this reconstitution scheme results in similar changes in

\[ \text{Adenylate Cyclase}\text{ (pM/min/mg UNC membranes)} \]

\[ \text{Extract Added (mg/mg UNC protein)} \]

\[ \text{B} \]

\[ \text{Adenylate Cyclase}\text{ (pM/min/mg UNC membranes)} \]

\[ \text{Extract Added (mg/mg UNC protein)} \]

\[ \text{B} \]
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basal and fluoride-stimulated activities, but this does not result in the restoration of a hormone response. To eliminate this effect of detergent, a second titration was performed in which the cholate concentration was kept constant. This was accomplished by supplementation of increasing amounts of the reconstituting extract with decreasing amounts of an extract that had been heated at 60°C for 15 min to destroy all reconstituting activity (Fig. 1B). Such titration results in a linear increase in hormone activation and a maintenance of a constant level of activity in the presence of fluoride. Activation of the enzyme by isoproterenol increased to a value that was 70% of that achieved with the halide (comparable to that with wild type membranes), and the effect of isoproterenol was completely blocked by the β-adrenergic antagonist propranolol. Saturation of the reconstitution of UNC membranes is emphasized in the inset to Fig. 1B. In this depiction fluoride-stimulated enzymatic activity is assumed to be a measure of the total adenylate cyclase activity of the UNC membrane, and relative recoupling is estimated as the ratio of stimulation by isoproterenol to that by fluoride. Percentage of stimulation by isoproterenol (compared to the basal rates) shows the same dependence on the addition of extract. A similar plot of the ratio of isoproterenol- to fluoride-stimulated activity from Fig. 1A (not shown) is sigmoidal with increasing amounts of extract, suggesting that an optimal amount of detergent stimulates reconstitution as well as adenylate cyclase activity.

Cholate extracts of wild type S49 cell membranes also restore PGE₂ stimulation of adenylate cyclase activity to UNC membranes (Table II), and reconstitution of activation by PGE₂, occurs coincidentally with that by isoproterenol. Stimulation by PGE₂ and isoproterenol was also restored in UNC membranes with extracts from membranes of HC-1, a cell line deficient in the catalytic subunit (C) of adenylate cyclase, and from B82, a cell line lacking β-adrenergic receptors. However, these responses were not restored when an extract of cyc⁻ membranes was used in the reconstitution; this confirms the lack of complementation between the UNC and cyc⁻ variants (11, 17).

Lubrol 12A9 extracts of various membranes were previously shown to contain the regulatory component of adenylate cyclase, G/F, and these extracts were capable of restoring adenylate cyclase activity to membranes of the cyc⁻ variant (4, 11); this activity was stimulated by β adrenergic agonists. Cholate extracts also have this capability (Table II; see also Fig. 11, Ref. 5). Reconstitution of basal and fluoride-stimulated activities with extracts of membranes from UNC, B82, HC-1, and wild type cells confirms the presence of G/F in those membranes. Recoupling of responses to both β adrenergic agonists and PGE₂ was also observed with all but the UNC extract.

While G/F and a coupling activity can be reconstituted using appropriate cholate extracts, neither the catalytic subunit, C, nor the β adrenergic receptor can be incorporated in an active state into deficient acceptor membranes with the procedures utilized to date. This is seen in the failure of a cyc⁻ extract (which had not been incubated at 23°C) to reconstitute the C-deficient HC-1 membrane or of a wild type extract to reconstitute a response to isoproterenol in membrane from B82 (Table II).

Properties of the Recoupled Hormone Receptor—Activation of adenylate cyclase in reconstituted UNC membranes shows essentially the same dependence on the concentration of isoproterenol as does that in wild type membranes (Fig. 2). Half-maximal stimulation occurred at 17 and 27 nM isoproterenol in wild type and reconstituted UNC membranes, respectively; reconstitution of cyc⁻ membranes yielded a Kₘ for isoproterenol of 19 nM (Table III). When stimulation by PGE₂ was examined, half maximal activities were observed at 125 and 190 nM with wild type and reconstituted cyc⁻ membranes. Slightly greater concentrations of the prostaglandin (Kₘ = 350 nM) were required with reconstituted UNC membranes.

When membranes from wild type S49 cells are exposed to guanine nucleotides, there is a decrease in the affinity of the

![Fig. 2. Activation of wild type and reconstituted UNC membranes as a function of hormone concentration. UNC membranes were reconstituted with wild type extract as described under "Experimental Procedures." Aliquots of these and wild type membranes were then assayed for adenylate cyclase activity in the presence of 50 µM GTP and the indicated amount of (-)-isoproterenol. For wild type membranes (○) the maximal response was achieved at an activity of 149 pmol·min⁻¹·mg⁻¹; the corresponding value for reconstituted UNC membranes (■) was 91 pmol·min⁻¹·mg⁻¹.](http://www.jbc.org/)

### Table II

Reconstitution of membranes deficient in hormone-stimulable adenylate cyclase with cholate extracts

| Source of Adenylate cyclase | Donor extract | Acceptor membranes | GTP | GTP + PGE₂ | GTP + INE | NaF | pmol/min/mg acceptor membrane |
|----------------------------|---------------|-------------------|-----|-----------|---------|-----|-----------------------------|
| UNC                        | UNC           | 10                | 24  | 12        | 284     |     |                             |
| B82                        | UNC           | 12                | 27  | 15        | 339     |     |                             |
| HC-1                       | UNC           | 15                | 200 | 232       | 274     |     |                             |
| Wild type                  | UNC           | 13                | 89  | 124       | 231     |     |                             |
| Wild type                  | UNC           | 16                | 147 | 181       | 265     |     |                             |
| B82                        | B82           | 12                | 104 | 190       | 116     |     |                             |
| Wild type                  | Wild type     | 50                | 41  | 71        | 81      |     |                             |
| Wild type                  | Wild type     | 48                | 106 | 95        |         |     |                             |
| UNC                        | UNC           | 7                 | 14  | 12        | 177     |     |                             |
| B82                        | B82           | 12                | 104 | 190       | 116     |     |                             |
| HC-1                       | HC-1          | 5                 | 41  | 71        | 81      |     |                             |
| Wild type                  | Wild type     | 8                 | 48  | 106       | 95      |     |                             |

*In this case the cyc⁻ extract was not warmed at 23°C to inactivate the catalytic component, C.*
beta-adrenergic receptor for agonists, but not for antagonists (9). Membranes from the two variants of S49, cyc- and UNC, have lost this capability (8, 9). They display only a single affinity that corresponds to the lower affinity of wild type (-)-propranolol, was approximately 10% of the total. 

**TABLE III**

**Hormone interaction with reconstituted membranes**

UNC and cyc- membranes were reconstituted with cholate extracts of wild type membranes as described under “Experimental Procedures.” Stimulation of adenylate cyclase by PGE, and isoproterenol was measured in the presence of 50 μm GTP. K_ach is the concentration of agonist required to achieve half-maximal stimulation. Binding was measured in the presence or absence of 50 μm GTP as described in Fig. 3. Dissociation constants (K_d) were calculated from the amount of isoproterenol (INE) required to inhibit [125I]iodohydroxybenzylpindolol binding by 50% as described (9).

| Membrane source          | Observed K_ach | K_d(INE) |
|--------------------------|----------------|----------|
|                          | PGE; GTP; GTP | GTP      |
| S49-wild type            | 125            | 70       | 360      |
| UNC                      | —              | 430      | 430      |
| UNC-reconstituted with heated extract | — | 390 | 390 |
| UNC-reconstituted         | 350            | 27       | 50       |
| cyc-                     | 550            | 550      |
| cyc- -reconstituted       | 150            | 19       | 135      |

**Table IV**

**Incubation requirements for cholate reconstitution**

A wild type cholate extract was used to reconstitute either UNC or cyc- membranes as described under “Experimental Procedures.” except for the indicated variations. Concentrations of GTP, isoproterenol (INE), Gpp(NH)p, and NaF during the assay of adenylate cyclase were 50 μm, 2 μm, 100 μm, and 10 mm, respectively.

**TABLE IV**

| Dilution | Second incubation | First incubation |
|----------|-------------------|------------------|
|          |                   |                  |
| A        | 10                | 10               |
| B        | 10                | 10               |
| C        | 10                | 10               |
| D        | 10                | 10               |

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low adenylate cyclase activity in cyc. Prominent loss of total enzymatic activity occurs with both types of membranes at temperatures above 20°C in the absence of Solution B. A second incubation at 30°C, following the addition of Solution B, results in the best activation by isoproterenol, although reconstitution of fluoride- and Gpp(NH)p-stimulated activity in cyc membranes shows little effect. A major requirement for Solution B at this stage appears to involve the stabilization of adenylate cyclase activity at 30°C, since its substitution with Solution A results in large losses of activity. If the initial incubation at 10°C is omitted prior to dilution and incubation in Solution B at 30°C, recoupling of UNC and reconstitution of cyc are decreased. Apparently, some time for equilibration at higher protein and detergent concentrations (and lower temperature) aids in reconstitution. Subsequent experiments have shown that a 20-min incubation on ice is sufficient.

The effects of the components of Solution B were also examined (Table V). ATP plays a major role in stabilization of adenylate cyclase activity, including that already present in the UNC acceptor membranes. The inclusion of GTP appears to cause an enhancement of all reconstituted enzymatic activities. Thus, when UNC membranes are reconstituted in the presence of GTP, hormone-stimulated activity is typically increased to a value that is 60 to 70% of that observed with fluoride. The relative importance of Mg++ is less clear, since both the membrane suspension and extract contained the cation. Therefore, its removal from Solution B left a concentration of 0.5 to 1.0 mM Mg++ in the reconstituting system. However, it should be noted that the concentration of ATP is in excess of that of residual Mg++; this condition is strongly inhibitory to adenylate cyclase catalytic activity.

In addition to the chemical constituents of Solution B, the dilution provided by its addition is important. If the ingredients in Solution B were added to the same final concentrations but in a smaller volume (such that there was little dilution, 15% versus 100%), membrane-bound enzymatic activity suffered severe losses (Table V). Dilution greater than the 2-fold utilized in the described procedure had no further beneficial effect.

Reconstitution of UNC Membranes Requires a Protein Component—The factor(s) responsible for the reconstitution of UNC membranes exhibits at least three properties that implicate or are consistent with the involvement of a protein in the process. First, the reconstituting activity of the cholera extract is very labile; a 30-min incubation at 37°C is sufficient to inactivate 75% of its reconstituting activity in UNC (Table VI). Similar lability is seen for the reconstitution of isoproterenol- and Gpp(NH)p-stimulated activity in cyc membranes, although the response to fluoride appears to be slightly more stable. (A similar or greater difference in the stability of G versus F activity has been seen previously with Lubrol extracts of G/F (3, 4).)

More convincing evidence comes from the inactivation of reconstituting activities with the sulphydryl reagent, N-ethylmaleimide. Both the hormone-stimulated activity (in UNC and cyc) and fluoride activation (in cyc) are inactivated by similar concentrations of the reagent (Table VI).

The sensitivity of the extract to digestion by protease is shown in Fig. 4. Reconstitution of stimulation by isoproterenol was about 10 times more sensitive to trypsin digestion than was the restoration of a fluoride response in cyc. Receptor reconstituting in both UNC and cyc showed identical sensitivity with trypsin. The intermediate sensitivity of stimulation of reconstituted cyc membranes by Gpp(NH)p is questionable. Reconstitution of responsiveness to hormone in UNC has an apparent stimulatory effect on activation of the enzyme by Gpp(NH)p (Fig. 4A); this effect is trypsin-sensitive, and part of the loss observed in Fig. 4B could be due to this phenomenon, rather than to actual damage to the G/F component. (These two possibilities could, however, be synonymous; see "Discussion").

Effect of Cholera Toxin-treated G/F on the Reconstitution...
Reconstitution of Membrane Function and Properties

Reconstitution of cyc- membranes with the extract from cholera toxin-treated HC-1 cells yields preparations that are so activated by GTP; a reduction in the level of response to fluoride is also characteristic. When the extract from toxin-treated cells was used to reconstitute UNC membranes, enzymatic activity that was markedly stimulated by GTP was also observed, although fluoride-stimulated activity was not reduced. This probably indicates that a mixture of both toxin-modified G/F and the original UNC G/F are interacting with the endogenous C of the UNC membrane.

DISCUSSION

We have established a new method for the reconstitution of membranes of S49 cell variants that are defective in hormone-sensitive adenylate cyclase activity; resultant membranes have properties that are essentially indistinguishable from those of wild type cells. UNC and cyc- membranes that have been reconstituted with cholate extracts from various membranes exhibit adenylate cyclase activity that can be stimulated by $\beta$-adrenergic agonists, PGE1, guanine nucleotide analogs, and fluoride. All of these activities are recoverable after collection of the reconstituted membranes by centrifugation. The resemblance between reconstituted and wild type membranes extends to their specific activities, to their dependence on the concentration of PGE1 or isoproterenol for enzyme activation, and to the $K_D$ value for binding of isoproterenol to $\beta$-adrenergic receptors. Thus, the ratio of $K_D$ to $K_{act}$ for the $\beta$-adrenergic agonist is very high with the reconstituted membranes (10; Table III); such values, which imply the ability to activate adenylate cyclase maximally despite minimal receptor occupancy, have previously been interpreted to be indicative of an efficiently coupled system (20, 21).

The alteration in the affinity of binding of $\beta$-adrenergic agonists that is characteristically observed in wild type membranes in the presence of guanine nucleotide is also observable in reconstituted UNC and cyc- membranes. However, the reconstitution of this phenomenon is not completely reproducible, despite the fact that reconstitution of responses to hormones is always observed. The reason for this variability is not known. Endogenous GTP (from membranes or extract) does not appear to be the problem, since reconstituted responses to hormones always show a similar dependence on added nucleotide (50%). This is lower than the 90 to 95% dependency of wild type membranes but is similar to the value of 70% seen in the same membranes after they are subjected to treatment with cholate as in a reconstitution protocol. Furthermore, this treatment with cholate does not interfere with the observation of nucleotide-induced changes in affinity of wild type membranes for $\beta$-adrenergic agonists. Inconsistency in the ability to observe guanine nucleotide-mediated changes in affinity could be indicative of relatively inefficient recoupling of receptors to a regulatory component of adenylate cyclase, despite a high value of $K_D/K_{act}$ and the possibility of efficient coupling of adenylate cyclase to receptors. One could envision a stimulation where all adenylate cyclase is subject to regulation by receptor, but where all receptor is not recoupled to the crucial component of the enzyme.

The method described above contrasts sharply with the previously reported reconstitution of cyc- membranes with extracts prepared with Lubrol 12A9 (4, 5, 11). In this case stable reconstitution was achieved only in the presence of certain activators of the enzyme system (i.e. Gpp(NH)p, fluoride, or GTP when extracts of cholera toxin-treated cells were used) (6). The essential irreversibility of these activating ligands prevented the subsequent examination of the effects
of other ligands after stable reconstitution had been achieved. There was little stable reconstitution of responsiveness to \(\beta\)-adrenergic agonists, although isoproterenol was effective in mixtures of membranes and extract. The effects of prostaglandins were minimal or nonexistent in membranes or in mixtures of membranes and extract. The latter result was probably due to interference from Lubrol that remained in the suspension of reconstituted membranes. The reasons for the markedly different results obtained with the two different detergents are not clear. Lubrol may have a greater affinity for factors that are necessary for reconstitution, such that a more specific interaction with acceptor membranes (presumably promoted by the activating ligands) and higher temperature are required to facilitate the movement of G/F into the membrane.

Also poorly understood are the improvements in the fidelity of reconstitution that are imparted by the specific procedures and conditions of the protocol (Tables IV and V). It is readily admitted that these evolved largely empirically; however, their existence provokes speculation. The protocol may be divided, simplistically, into three phases: (a) incubation at low temperature and high concentrations of membranes and extract-containing detergent; (b) dilution; and (c) incubation at higher temperature. We suggest that the first incubation at high concentrations of soluble factors and membranes facilitates their initial interaction. An optimal concentration of cholate at this stage might provide beneficial perturbation of the acceptor bilayer. The data of Fig. 1 and other attempts to manipulate detergent concentration at this stage (not shown) are consistent with the hypothesis that cholate facilitates reconstitution. Low temperature is essential at this stage to prevent denaturation of the catalytic moiety of adenylate cyclase at elevated temperature and high concentration of cholate. Subsequent dilution (Stage 2) prior to elevation of temperature is then necessary to lower the cholate concentration. Interestingly, while solubilization is achieved at concentrations of cholate that are above the critical micellar concentration, optimal reconstitution is obtained when the detergent concentration in Stage 1 is near to or slightly below this value (22, 23). Reconstitution at this stage is stimulated greatly by the use of Solution B. Presumably the addition of CTP and ATP promotes the interaction of factors with acceptor membranes. Elevation of temperature, the third stage, appears to facilitate the stable incorporation of factors into the membrane, perhaps in part because of necessary alteration of the lipid structure. Inclusion of ATP at this stage stabilizes adenylate cyclase against detergent-facilitated denaturation. Further speculation is limited by our current necessary reliance on determinations of enzymatic activity to assess the status of individual components of the system during their reconstitution.

A major question involves the nature of the lesion in the UNC variant. Previous analysis has demonstrated that the gross lipid composition of the UNC membrane is identical with that of wild type and that there is an identical electrophoretic pattern of the major membrane proteins (24). The reconstitution procedure described herein was thus designed to help answer this question. To date, we can say that the lesion is correctable by the appropriate addition of one or more factors and that a crucial factor is temperature-sensitive and susceptible to inactivation by N-ethylmaleimide and by trypsin. Furthermore, initial data (not shown), obtained by ultracentrifugation of cholate extracts through sucrose gradients, are consistent with the residence of UNC-reconstituting activity in a fraction with a molecular weight of approximately \(10^5\) (similar to that of C/F). These data seem to indicate the involvement of at least one protein in the reconstitution of UNC membranes. This protein might be either a new component of the adenylate cyclase system or one of the three components already identified, receptor, G/F, or C.

Considerable evidence indicates that the lesion does not involve the receptor. The defect in UNC membranes results in the loss of responses to both PGE and \(\beta\)-adrenergic agonists, and, therefore, it presumably involves a common link through which both types of receptors interact with adenylate cyclase. Also consistent with this reasoning is the fact that reconstitution of UNC membranes with cholate extract restores both responses in parallel. Furthermore, the procedures utilized to date have failed to reconstitute responses to \(\beta\)-adrenergic agonists in receptor-deficient membranes (Table II). Finally, examination of responses to isoproterenol in heterokaryons formed by the fusion of UNC or cyc~ S49 cells with B25 (in the presence of cycloheximide) indicates that both S49 cell variants contain functional \(\beta\)-adrenergic receptors (17). The remaining receptor-related hypothesis is that the receptor for PGE, is reconstituted and that it serves as an essential coupling factor for the \(\beta\)-adrenergic receptor. There is no reason to consider this a likely possibility.

The catalytic subunit of adenylate cyclase, C, is also not a good candidate for the site of the UNC defect. In addition to the fact that UNC has normal basal and adenylate cyclase activity, cholate extracts containing C do not restore enzymatic activity to C-deficient membranes (Table II). Furthermore, the reconstitution of UNC acceptor membranes is routinely accomplished with extracts in which C activity has been destroyed by warming or with extracts from C-deficient cells (Table II), and, if UNC acceptor membranes are incubated at 37°C prior to reconstitution, the restoration of response to hormone is reduced in parallel with the loss of UNC catalytic activity (not shown).

A defect in the regulatory component of adenylate cyclase, G/F, remains as an excellent possibility to explain the deficiency of UNC, despite the existence of Gpp(NH)p and fluoride-stimulated cyclase activity in these membranes. The following observations are consistent with this hypothesis: (a) recoupling activity is as stable as or is more labile than G/F activity when both are inactivated by temperature, N-ethylmaleimide, or proteases; (b) initial experiments indicate co fractionation of UNC and cyc~ reconstituting activities by gel filtration or sucrose density gradient centrifugation; (c) UNC and cyc~ (G/F-deficient) variants are not complementary in vitro (Table II; Refs. 11 and 17); and (d) G/F activity is probably incorporated into UNC membranes during the procedure utilized to reconstitute them, and this G/F may function in tandem with endogenous C (Table VII). These observations are consistent with the notion that there exists a domain of G/F that is crucial for responsiveness to hormones.

It is reasonable to speculate that this is a region of the protein that is subject to post-translational modification and that the system necessary for such modification is altered in the UNC variant. (The high frequency of the UNC lesion and the constancy of the phenotype perhaps argue against a specific alteration in primary amino acid sequence of C/F.)

However, the observations just stated are also completely consistent with the deficiency in UNC of an unidentified central component of the adenylate cyclase system. While lack of complementation between UNC and cyc~ and co-fractionation and co inactivation of recoupling and G/F activities tend to suggest linkage of function, this linkage could be noncovalent. The existence of a coupling factor that is normally tightly bound to G/F would explain the coincidence of behavior and the lack of complementation with cyc~ if G/F is necessary, for example, for the insertion of the factor into the membrane. We cannot yet distinguish between these two possibilities or the somewhat less likely possibility of a com-
pletely independent component. Purification of G/F and of UNC-reconstituting activities will obviously provide the answer.

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