Vol. 147, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1987 Pages 443-451

RECONSTITUTION OF THE G_S PROTEIN FROM B16 MELANOMA CLONES OF HIGH AND LOW EXPERIMENTAL METASTATIC POTENTIAL INTO S49 CYC⁻ MEMBRANES

Bruce R. Lester[1,3], Jeffrey M. Stadel[2], Charles Buscarino[1]
John Sheppard[4], Russell G. Greig[1], and George Poste[1]

Departments of Cell Biology[1] and Molecular Pharmacology[2],
Smith Kline & French Laboratories,
1500 Spring Garden Street,
Philadelphia, PA 19101

Department of Laboratory Medicine and Pathology[3],
and Genetics and Cell Biology[4],
Dight Laboratories,
University of Minnesota,
400 Church St., S.E.,
Minneapolis, MN 55455

Received July 27, 1987

SUMMARY: The ability of a series of B16 melanoma clones to form experimental lung metastases in syngeneic mice has been shown to correlate positively with adenylate cyclase activity. (Sheppard et al, Int. J. Cancer 37 (1986) 713-722). To begin to identify the components of the adenylate cyclase complex that account for enhanced enzyme activity in highly metastatic tumor populations, cholate extracts containing the GTP-binding protein G_S from B16 melanoma clones of different metastatic capacities were reconstituted with membranes prepared from S49 cyc⁻, a variant lymphoma cell line that lacks G_S function. The results revealed that extracts from a highly metastatic B16 clone (F10-C23) reconstituted significantly greater adenylate cyclase activities in S49 cyc⁻ membranes than parallel preparations from a B16 clone (F1-C29) of low metastatic capacity. The data suggest that aberrations in G_S function may contribute to the heightened responsiveness of adenylate cyclase observed in B16 melanoma clones of increased metastatic potential.

The pharmacology of tumor cell dissemination and the hormonal mechanisms that may influence the different stages of the metastatic process are poorly understood. In previous investigations on a murine tumor model, we had

The abbreviations used are: BSA, bovine serum albumin; Gpp(NH)p, 5'-guanyl-ß-γ-imidodiphosphate; MSH, melanocyte stimulating hormone; G_S, stimulatory guanine nucleotide regulatory protein of the adenylate cyclase system; SDS, sodium dodecylsulfate.

0006-291X/87 $1.50

established that the ability of mouse B16 melanoma clones to form lung colonies after i.v. inoculation (experimental metastases) correlates positively with their ability to accumulate cAMP when challenged with agents that activate adenylate cyclase (e.g., forskolin or MSH) (1). Biochemical investigations on isolated plasma membranes revealed that B16 melanoma clones of high metastatic potential consistently displayed a greater than 10-fold activation of adenylate cyclase when stimulated directly by the diterpene forskolin (2). This is in contrast to the adenylate cyclase of B16 melanoma clones of low metastatic capacity which exhibited a less than 5-fold activation under identical conditions. In the course of the same studies, we also observed that a combination of forskolin and the nonhydrolyzable GTP analog, Gpp(NH)p, produced a synergistic activation of the adenylate cyclase in the high metastatic clones but the effect of these agents was less than additive on the cyclase from low metastatic cells.

Both forskolin and Gpp(NH)p are thought to activate adenylate cyclase by modulating the interaction of G_s with the catalytic unit of the enzyme (3-9). At high concentrations, forskolin has also been shown to stimulate directly the catalytic component (10-12). Our previous data suggested that aberrations in G_s coupling to the catalytic unit of adenylate cyclase contributed to differences in cAMP metabolism observed in B16 metastatic variants (2). In the present investigations, we have attempted to examine this possibility by selectively measuring the G_s activity of B16 clones using reconstitution studies. This was achieved by cholate extraction of G_s from B16 plasma membranes followed by reconstitution with the adenylate cyclase catalytic unit in plasma membranes derived from S49 cyc^- lymphoma cells (13), a cell line that lacks the G_s protein (14). The results revealed that the biochemical phenotype associated with high and low B16 metastatic potential can be transferred via the reconstitution procedures and suggest that aberrations in adenylate cyclase activity associated with enhanced expression of B16 metastatic properties may be related to alterations in G_s function.

MATERIALS AND METHODS

Cell Culture

The origin and properties of the B16-F1 and B16-F10 melanoma sublines have been described (15). The B16 melanoma F10-C23 and F1-C29 clones were isolated by the limiting dilution technique and maintained in culture according to published procedures (16).

Animals

Female C57BL/6 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA) and the Laboratory Animal Services Division of Smith Kline & French Laboratories (Philadelphia, PA). Animals were age- and sex-matched within each experiment.

Experimental Pulmonary Metastasis

Metastatic potential was assayed as described (1). The B16 clone F10-C23 formed >300 lung colonies following an intravenous inoculum of 5×10^4 cells and was defined as expressing high metastatic potential. Under identical conditions, clone F1-C29 gave rise to a median of only three lung colonies and was classified as having low metastatic capacity. A complete description of metastatic properties of these two clones have been described previously (1,2,17).

Preparation of plasma membranes from B16 melanoma clones

Membranes were prepared as described (2).

Reconstitution

Reconstitution of Mg-ATP-dependent adenylate cyclase in S49 cyc^- was performed according to Sternweis and Gilman (13). Briefly S49 cyc^- plasma membranes were prepared as described above and the final pellet resuspended in HEPES buffer (protein concentration = 1.0 mg/ml). B16 plasma membranes were suspended in 1% cholate (protein concentration = 5.0 mg/ml) and stirred on ice for 1 hr to solubilize G_s. Following centrifugation (100,000g x 20 min.), the pellet was resuspended in HEPES buffer (protein concentration = 1.0 mg/ml) and assayed for adenylate cyclase. Cholate extracts were incubated at 30°C for 20 min to inactivate residual catalytic component and then mixed with S49 cyc^- membranes at a final cholate concentration of 0.36%. To ensure saturation of S49 cyc^- membranes with B16 melanoma G_s, cholate extracts were diluted with 1% cholate in HEPES buffer to provide samples constant in cholate concentration but containing decreasing amounts of G_s. Following reconstitution, membranes were centrifuged (100,000g x 20 min.), the pellets resuspended in HEPES buffer and assayed for adenylate cyclase activity.

Adenylate Cyclase Assay

Adenylate cyclase activity was assayed as described previously (2).

Protein Determination

Protein was measured by the method of Lowry *et al* (19) using BSA as a standard.

[^{32}P]ADP-ribosylation of G_s

G_s of membranes prepared from the B16 melanoma clones was labeled in the presence of [^{32}P]NAD (5μM) and cholera toxin (50 μg/ml) as described

previously (20). [^{32}P]ADP-ribosylated G_s was isolated by SDS-polyacrylamide gel electrophoresis (21) after equal amounts of membrane protein (14 μg) from each B16 clone were loaded onto the gel. The [^{32}P]-labeled G_s was located on the gel by autoradiography, the protein band excised and quantitated by scintillation counting.

MATERIALS

BSA, cAMP, ATP, creatine phosphate, creatine phosphokinase, EGTA, GTP, Gpp(NH)P, Norit-SGX charcoal and MSH and IBMX were purchased from Sigma Chemical Co. (St. Louis, MO). Neutral aluminum oxide was obtained from E. Merck, Elmsford, NY, Dowex AG 50W-X8 from Bio-Rad Laboratories, Richmond, CA; α[^{32}P]ATP (20 Ci/mmole) from Amersham/Searle Corporation, Des Plains, IL; [^{3}H]cAMP (30 Ci/mmole) from New England Nuclear, Boston, MA; and forskolin from Calbiochem, La Jolla, CA.

RESULTS

The responsiveness of adenylate cyclase in plasma membranes derived from B16 melanoma clones of high (F10-C23) and low (F1-C29) metastatic capacity to different stimuli is shown in Figure 1. Gpp(NH)p elicited comparable

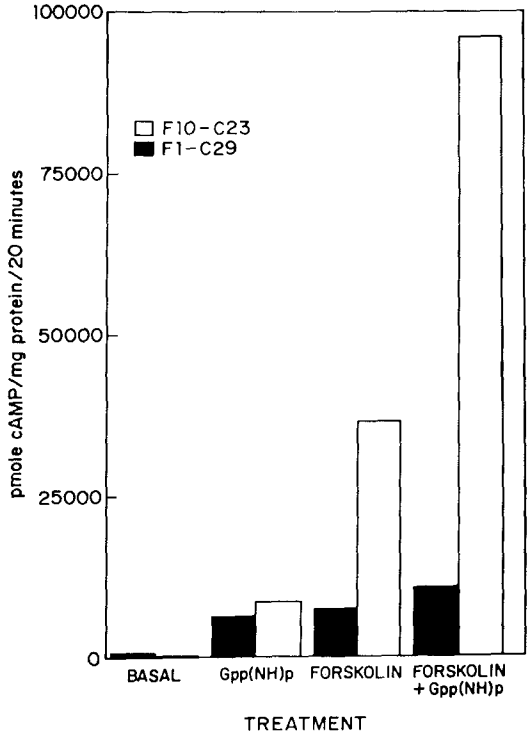

Figure 1. Activation of adenylate cyclase in B16 melanoma clones of low (F1-C29) and high (F10-C23) experimental metastatic potential. Partially purified membranes were challenged with Gpp(NH)p (10 μM), forskolin (100 μM) or forskolin (100 μM) plus Gpp(NH)p (10 μM) for 20 min at 30°C. Adenylate cyclase activity was determined as described in Materials and Methods.

responses in membranes isolated from the two clones. In contrast, forskolin was more effective in stimulating adenylate cyclase in membranes from F10-C23 cells than in parallel preparations from F1-C29 cells. The combination of Gpp(NH)p and forskolin induced a synergistic response in the adenylate cyclase in the F10-C23 cells but the response in the low metastatic F1-C29 clone was less than additive.

To investigate further the marked difference in the adenylate cyclase activities of the two clones, we next attempted to resolve the components of the adenylate cyclase complex. The G_s proteins from the F10-C23 and F1-C29 clones were covalently labeled in the presence of [^{32}P]NAD and cholera toxin (20). Following SDS-polyacrylamide gel electrophoresis we found that the pattern of [^{32}P]ADP-ribosylated G_s was identical for the two clones ($M_r \simeq 45,000$) and the incorporation of label into G_s was not significantly different (data not shown).

The functional properties of G_s from the B16 melanoma clones were examined by reconstitution techniques. Following procedures developed by Sternweis and Gilman (13), the G_s proteins from the plasma membranes of the F10-C23 and F1-C29 clones were solubilized by cholate extraction. Incubation of the detergent extracts for 20 min at 30°C inactivated residual adenylate cyclase catalytic unit, as monitored by challenge with 0.1 mM forskolin (data not shown), thus eliminating its contribution to adenylate cyclase activities measured in the reconstitution system. The G_s in the cholate extracts was subsequently reconstituted with the catalytic unit of adenylate cyclase in S49 cyc$^-$ membranes (Figure 2). As reported previously (22) the adenylate cyclase activity of naive S49 cyc$^-$ membranes was stimulated by forskolin but not Gpp(NH)p. Also, synergistic activation of the cyclase in the combined presence of Gpp(NH)p and forskolin was not observed. However, reconstitution with G_s from either F10-C23 or F1-C29 cells resulted in activation of adenylate cyclase by Gpp(NH)p comparable to that seen in the relevant B16 donor clones. Following reconstitution with the G_s extract from the high metastatic F10-C23 clone an increased responsiveness of the

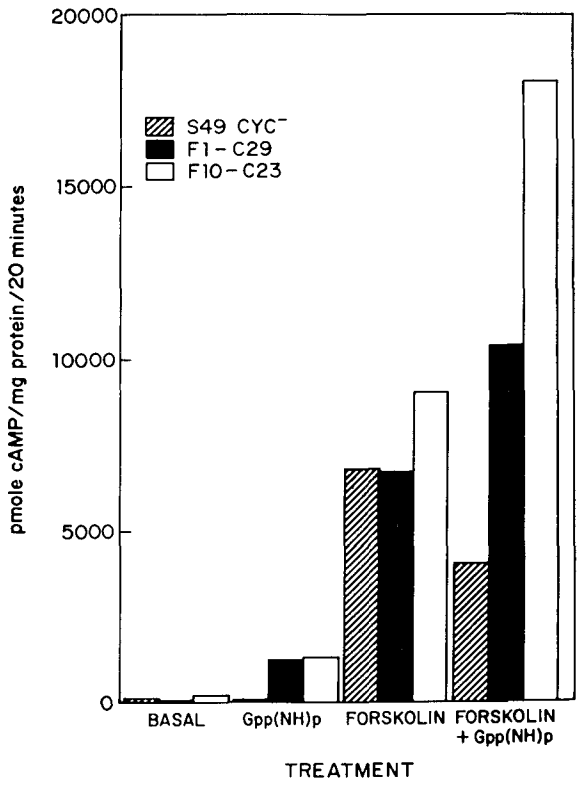

Figure 2. Reconstitution of adenylate cyclase activity in S49 cyc⁻ membranes. Cholate extracts from the B16 melanoma clones F1-C29 and F10-C23 were reconstituted with S49 cyc⁻ membranes and adenylate cyclase activity measured as indicated.

adenylate cyclase to forskolin alone was observed relative to naive S49 cyc⁻ membranes and a synergistic response was detected in the reconstituted system when challenged with a combination of Gpp(NH)p and forskolin. In contrast, G_s extracts from low metastatic F1-C29 clone were significantly less effective. S49 cyc⁻ membranes reconstituted with G_s extracts from this clone failed to show enhanced responsiveness to forskolin, and enzyme activity in the presence of both forskolin and Gpp(NH)p was only slightly greater than additive. Moreover, the extract from F1-C29 membranes inhibited the reconstitution of adenylate cyclase activity by the extract from F10-C23 membranes. Stimulation of S49 cyc⁻ adenylate cyclase by the combination of forskolin and Gpp(NH)p following reconstitution with extracts of F10-C23 membranes, F1-C29 membranes or with a mixture of both extracts (1:1) were 134-fold, 50-fold and 94-fold

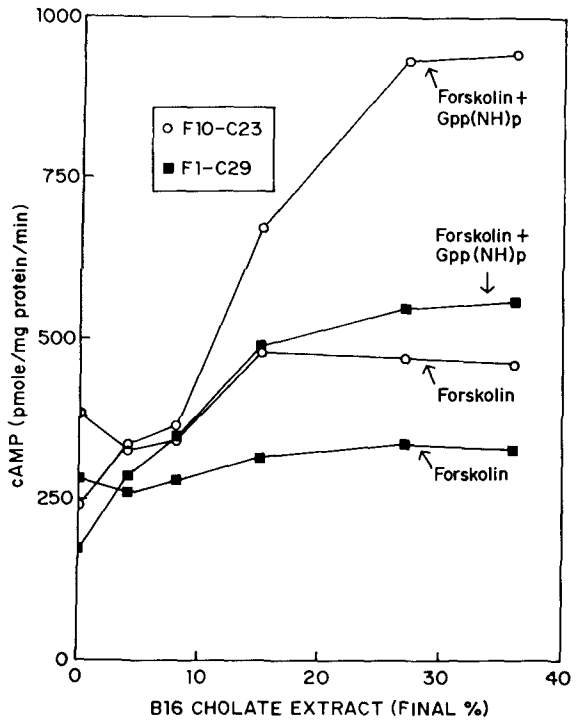

Figure 3. Titration of S49 cyc⁻ membranes with cholate extracts from the B16 melanoma clones, F1-C29 (■) and F10-C23 (O). Cholate extracts were incubated with S49 cyc⁻ membranes and the reconstituted membranes challenged with forskolin 100 μM or a combination of forskolin (100 μM) and Gpp(NH)p (10 μM) as indicated.

respectively. The results of these experiments indicate that the properties of the reconstituted adenylate cyclase mirror the activities of the donor B16 melanoma clones of distinct metastatic potentials.

To eliminate the possibility that the differences in adenylate cyclase responsiveness in the reconstituted S49 cyc⁻ membranes were due to an artifact in the differential extraction and transfer of G_s from the B16 clones, a reconstitution titration was carried out in which increasing concentrations of cholate extract from F10-C23 and F1-C29 clones were combined with a fixed amount of S49 cyc⁻ membranes. The detergent concentration was maintained constant at 0.36% during the titration. As shown in Figure 3, the responses of the reconstituted adenylate cyclase to either forskolin or the combination of forskolin and Gpp(NH)p saturated with respect to the G_s extracts from both the F10-C23 and F1-C29 clones.

DISCUSSION

In the present study, enzyme reconstitution studies using the adenylate cyclase from membranes of S49 cyc^- cells were used to assess G_s activities in murine B16 melanoma clones of low (F1-C29) and high (F10-C23) metastatic capacity. The results indicated that G_s extracted from both F1-C29 and F10-C23 clones can reconstitute adenylate cyclase activity in S49 cyc^- membranes and that the level of enzyme activity reflects the enzyme properties of the donor melanoma cells. These conclusions are supported by the following observations. First, reconstitution with G_s extracted from either F1-C29 or F10-C23 clones conferred Gpp(NH)p-sensitive adenylate cyclase activity to the S49 cyc^- membranes. Stimulation of the S49 cyc^- cyclase by NaF and the beta-adrenergic agonist isoprotenenol were also simultaneously reconstituted (data not shown). However, since these parameters do not correlate with metastatic potential (1,2) they were not further characterized. These data are consistent with donation of G_s activity to S49 cyc^- membranes from the B16 melanoma clones.

Second, S49 cyc^- membranes reconstituted with extracts from the highly metastatic F10-C23 clone exhibited significantly greater synergistic activation of adenylate cyclase when challenged with a combination of Gpp(NH)p and forskolin than membranes reconstituted with extracts from F1-C29, a clone of low metastatic potential. These differences in reconstituted adenylate cyclase activities were not attributable to differences in the amount of G_s in the two B16 clones. Although we have studied only two clones, the results suggest that enhanced G_s activity is associated with augmented metastatic capacity. One caveat in the interpretation of these observations is that although activation of adenylate cyclase by a combination of Gpp(NH)p and forskolin in donor F1-C29 membranes was, as expected, less than additive in the reconstituted S49 cyc^- preparations, the effects of these two agents was slightly greater than additive. The reason for this apparent discrepancy is not clear, but may reflect imprecisions inherent in the reconstitution procedures.

The role of cAMP in unregulated cell proliferation and metastatic spread remains to be defined. Previous studies have linked these processes with alterations in adenylate cyclase activities and modifications of adenylate cyclase-associated guanine nucleotide binding proteins (23-25). Our results provide evidence that aberrations in G_s function contribute to the heightened responsiveness of adenylate cyclase in B16 melanoma clones expressing enhanced metastatic potential.

ACKNOWLEDGMENTS

We thank JoAnn Schultz for excellent secretarial assistance.

REFERENCES

1. Sheppard, J., Koestler, R., Corwin, S., Buscarino, C., Doll, J., Lester, B., Greig, R. and Poste, G. (1984) Nature 308, 544-547
2. Sheppard, J.R., Lester, B., Doll, J., Buscarino, C., Gonzales, E., Corwin, S., Greig, R. and Poste, G. (1986) Internatl. J. Canc. 37, 713-722
3. Sibley, D.R. and Lefkowitz, R.J. (1985) Nature 317, 124-129
4. Lefkowitz, R.J., Stadel, J.M. and Caron, M.G. (1983) Ann. Rev. Biochem. 52, 159-186
5. Jakobs, J.H., Aktories, K. and Schultz, G. (1983) Nature 303, 177-178
6. Linger, Z. and Cassel, D. (1981) Adv. Cyc. Nucl. Res. 14, 15-22
7. Sternweis, P.C. and Gilman, A.G. (1982) Proc. Natl. Acad. Sci. USA 78, 4888-4891
8. Awad, J.A., Johnson, R.A., Jakobs, K.H. and Schultz, G. (1983) J. Biol. Chem. 258, 2960-2965
9. Seamon, K. and Daly, J.W. (1985) Adv. in Cyc. Nucl. Res. 19, 125-135
10. Seamon, K.B., Padgett, W. and Daly, J.W. (1981) Proc. Natl. Acad. Sci. USA 79, 3363-3367
11. Van Sande, J., Cochaux, P., Mockel, J. and Dumont, J.E. (1983) Molec. Cell. Endocrine 29, 109-119
12. Siegl, A.M., Daly, J.W. and Smith J.B. (1982) Mol. Pharm. 21, 680-687
13. Sternweis, P.C. and Gilman, A.G. (1979) J. Biol. Chem. 254, 3333-3340
14. Gilman, A.G. (1984) J. Clin. Invest. 73, 1-4
15. Fidler, I.J. (1973) Nature New Biol. 242, 148-149
16. Poste, G., Doll, J., Brown, A.E., Tzeung, J. and Zeidman, I. (1982) Cancer Res. 42, 2770-2778
17. Lester, B.R., Greig, R.G., Buscarino, C., Sheppard, J.R., Corwin, S.P. and Poste, G. (1986) Int. J. Cancer 38, 405-411
18. Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541-548
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R. (1951) J. Biol. Chem. 193, 265-275
20. Stiles, G.L., Stadel, J.M., DeLean, A., and Lefkowitz, R.J. (1981) J. Clin. Invest. 68, 1450-1455.
21. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
22. Seamon, K. and Daly, J.W. (1981) J. Biol. Chem. 256(19), 9799-9801
23. Burchiel, S.W., Hanson, K. and Warner, N. (1984) Int. J. Immunopharmac. 6(1), 35-42
24. Clark, O.H., Gerend, P.L. and Nissenson, R.A. (1984) World J. Surg. 8, 466-473
25. Woolkalis, M.J., Nakada, M.T. and Manning, D.R. (1986) J. Biol. Chem. 261(7), 3408-3413