Monoclonal antibodies to the guanine-nucleotide binding proteins of adenylate cyclase

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Monoclonal antibodies (Mabs) to the stimulatory (N_s) and inhibitory (N_i) guanine nucleotide regulatory proteins associated with adenylate cyclase have been developed. Two Mabs (2A₃ and 5G₁₂), which are of the IgG_{2b} subclass, recognize the β -subunits (β) of N_s , N_i and transducin. Iodinated β can be immunoprecipitated by either Mab coupled to Affi-Gel 10 and this can be decreased by prior incubation of the Mabs with excess unlabelled β . The Mabs stabilize the activated state of N_s while decreasing the rate of deactivation of activated N_s in the presence of β .

INTRODUCTION

The hormone-sensitive adenylate cyclase system consists of three proteins: (i) a catalytic subunit (C) that converts ATP into cyclic AMP; (ii) a specific receptor binding site (R) for hormones and neurotransmitters; and (iii) a stimulatory (N_s) or inhibitory (N_i) guanine-nucleotide regulatory protein that couples R to C. Stimulation of adenylate cyclase is mediated through N_s , which can be ADP-ribosylated by cholera toxin and hydrolyses GTP to GDP after hormonal stimulation of adenylate cyclase (Brandt *et al.*, 1983; Cassel & Selinger, 1978; Northup *et al.*, 1982; Pfeuffer, 1977). Inhibition of adenylate cyclase is mediated through N_i , which can be ADP-ribosylated by pertussis toxin (Bockoch *et al.*, 1983; Katada & Ui, 1981). Available evidence suggests that receptor-mediated stimulation and inhibition of adenylate cyclase occurs when the subunits of N_s and N_i dissociate (Northup *et al.*, 1982, 1983a,b; Katada *et al.*, 1984a,b,c).

The question of how the subunits of N_s and N_i interact with each other and with receptors and C must be addressed if we are to understand how hormones regulate adenylate cyclase activity. To approach this question we have prepared Mabs to N_s . Mabs were raised with the aim of producing defined immunological reagents of high specificity which could be used as probes to study the structure and function of the guanine-nucleotide binding proteins.

In the present paper we describe the development of Mabs, two of which are directed towards the β -subunits of N_s , N_i and transducin, the latter being a guanine nucleotide binding protein present in the retina that is structurally homologous with N_s and N_i . The Mabs, in the presence of excess β , decrease the rate of deactivation of activated N_s , while stabilizing the activated state of N_s in the absence of excess β .

MATERIALS AND METHODS

Materials

Rabbit livers were obtained from Pel-Freez (Rogers, AR, U.S.A.). Heptylamine-Sepharose was synthesized by the method of Shaltiel (1974) with modifications as described by Northup *et al.* (1980). Sodium cholate was obtained from Sigma and purified as described by Northup *et al.* (1980). Na ¹²⁵I was obtained from Amersham (Arlington Heights, IL, U.S.A.) while [α -³²P]ATP was purchased from ICN (Irvine, CA, U.S.A.). Affi-Gel 10 and all the reagents used for analytical SDS/polyacrylamide-gel electrophoreses were obtained from Bio-Rad (Richmond, CA, U.S.A.). Nitrocellulose filter paper (0.4 μ m) was obtained from Bio-Rad. All other chemicals were of reagent grade.

Purification of N_s and β

N_s was purified from liver as described by Sternweis *et al.* (1981) while free β was obtained from the second DEAE-Sephacel and the hydroxyapatite columns as described by Northup *et al.* (1983a).

Assays

N_s and β activities were measured as described by Sternweis *et al.* (1981) and by Northup *et al.* (1983a) respectively. Protein was determined by the procedures of Schaffner & Weismann (1973) or Bradford (1976). SDS/polyacrylamide-gel electrophoresis was carried out as described by Laemmli (1970) and gels were stained with silver as described by Wray *et al.* (1981).

Immunization of mice and fusion

Female Balb/c mice were immunized with 50 μ g of N_s per injection at 2-week intervals, over a period of 2 months. At 7 days prior to fusion, the mice were boosted intravenously with 25–50 μ g of N_s , resuspended in

Abbreviations used: N_s , the stimulatory GTP-binding regulatory component of adenylate cyclase; N_i , the inhibitory GTP-binding regulatory component of adenylate cyclase; β , the β -subunits of N_s and N_i ; T, transducin (the GTP-binding regulatory component from retina); Mabs, monoclonal antibodies; e.l.i.s.a., enzyme-linked immunosorbent assay.

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20 mM-Tris/HCl, pH 8.0, containing 1 mM-EDTA, 1 mM-dithiothreitol, 100 mM-NaCl and 0.1% Lubrol, daily for 5 days. After a 2-day rest period, the mice were killed and spleens were aseptically removed for fusion on the seventh day. Spleen cells were fused with P3 $\times$ 63-Ag8 myeloma cells, and hybridomas were isolated as described by St. Groth & Scheidegger (1980).

E.l.i.s.a.

Hybrids were screened by the e.l.i.s.a. method as described by Brown & Juliano (1985). Supernatants that gave a reading of at least 2–3-fold above background were expanded, cloned repeatedly (at least three times) and then injected into 2,6,10,14-tetramethylpentadecane (pristane) primed mice for ascites, which were collected within 2 weeks.

Iodination of protein

N_s and free β were iodinated by using the chloramine-T procedure (Oi & Herzenberg, 1980). Iodinated protein was separated from free iodine by gel filtration over a 15 ml Sephadex G-25 (medium grade) column equilibrated with 20 mM-Tris/HCl, pH 8.0, containing 1 mM-EDTA, 1 mM-dithiothreitol, 100 mM-NaCl, 0.1% Lubrol and 0.1% gelatin. Iodinated protein was eluted in the void volume and more than 90% of the counts were precipitated by 15% (w/v) trichloroacetic acid.

Coupling of antibodies to Affi-Gel 10

The IgG fraction of ascites fluid, obtained by $(NH_4)_2SO_4$ precipitation (Jonak, 1980), was dialysed against phosphate-buffered saline, pH 7.5, diluted to 10 mg/ml and coupled to Affi-Gel 10 according to the procedure supplied by Bio-Rad. More than 80% of the antibody was coupled to the gel.

Immunoprecipitation of iodinated proteins

Iodinated proteins were immunoprecipitated with Mabs coupled to Affi-Gel 10. Briefly, $(1-2) \times 10^6$ c.p.m. plus the antibody-Affi-Gel 10 mixture were incubated at 4 °C overnight. Immune complexes were sedimented by centrifugation and repeatedly washed (at least four times) with 50 mM-Tris/HCl, pH 8.0, containing 150 mM-NaCl, 1 mM-EDTA, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.2% gelatin and 0.1% NaN_3 . Samples were electrophoresed on 11% SDS/polyacrylamide gels, silver-stained, dried and exposed to Kodak X-ray film (XAR 5) in the presence of intensifying screens at -70 °C.

Electroblotting and immunological detection of proteins on nitrocellulose

Proteins were transferred from SDS/polyacrylamide gels to nitrocellulose filter paper as described by Towbin *et al.* (1979). Electrophoretic blots, which were soaked in 3% gelatin/20 mM-Tris/HCl (pH 7.5)/500 mM-NaCl to saturate additional protein binding sites, were incubated with Mabs diluted in 1% gelatin/20 mM-Tris/HCl (pH 7.5)/500 mM-NaCl. Filters were washed and incubated with goat anti-(mouse IgG) coupled to horseradish peroxidase (Bio-Rad). Antigen-antibody complexes were detected with 0.05% 4-chloro-1-naphthol and 0.015% H_2O_2 in 20 mM-Tris/HCl (pH 7.5)/500 mM-NaCl. The reaction was terminated with distilled water and the filters were dried and stored protected from light.

Fig. 1. Immunoprecipitation of N_s , β and T_β by Mabs $2A_3$ and $5G_{12}$.

N_s , β and T_β were iodinated with chloramine-T and $2A_3$ or $5G_{12}$ were coupled to Affi-Gel 10 as described in the Materials and methods section. Immune complexes, which were formed overnight at 4 °C, were repeatedly washed and electrophoresed on 11% SDS/polyacrylamide gels as described by Laemmli (1970). Gels were fixed in 50% methanol, stained with silver (Wray *et al.*, 1981), dried down and placed next to Kodak X-ray film (XAR-5) at -70 °C. Lanes 1 and 2 are samples of iodinated N_s (lane 1) and β (lane 2). Immune complexes using N_s (lanes 3 and 4), β (lanes 5 and 6) and T_β (lanes 7, 8 and 9) in the absence (lanes 3, 5 and 9) or in the presence (lanes 4, 6, 7 and 8) of Mabs $2A_3$ (lanes 4, 6 and 7) or $5G_{12}$ (lane 8) are also shown. Transducin was generously supplied by Stuart Critz.

Test for antibody class

The class and the subclass of the heavy chain of the Mabs were determined by the Ouchterlony (1949) technique.

RESULTS

The question of how the subunits of N_s and N_i interact with each other and R and C must be addressed if we are to understand how hormones regulate adenylate cyclase activity. We approached this question by developing Mabs to the guanine nucleotide regulatory proteins with the aim of producing specific immunological reagents that could be used as probes to study the structure and function of the regulatory proteins.

At 2–3 weeks after plating out the fusion products, culture supernatants were assayed for Mabs directed against the guanine nucleotide regulatory proteins by using the e.l.i.s.a. Of the wells from the fusion, 3% (16/480) were positive and two colonies, designated as $2A_3$ and $5G_{12}$, were cloned at least three times by limiting dilution. Characterization of the Mabs using class- and subclass-specific rabbit anti-(mouse IgG) indicated that the Mabs were of the IgG_{2b} subclass.

The data from the e.l.i.s.a. indicated that the Mabs recognized an antigenic determinant in the admixture of proteins on the microtitre plate, but gave no indication as to whether the Mabs were directed towards N_s and its constituent subunits, or to another unrelated protein that co-purified with N_s . To address this question we immunoprecipitated iodinated N_s or free β using $2A_3$ and $5G_{12}$ coupled to Affi-Gel 10; the results using $2A_3$ are shown in Fig. 1. $2A_3$ coupled to Affi-Gel 10 recognized

Fig. 2. Immunoblot of β with Mab 2A₃ or control antibody

β (at 1 μ g/lane) was electrophoresed and transferred to nitrocellulose filter paper as described. The paper was blocked with 3% gelatin and incubated with primary antibody [1:300 dilution of ascites fluid which has been precipitated with 50% (NH₄)₂SO₄ and dialysed against phosphate-buffered saline, pH 7.5] for several hours. If purified antibody was used the antibody concentration was 0.5–1.0 μ g/ml and the incubation was carried out overnight. Immune complexes were detected with affinity purified goat anti-(mouse IgG) coupled to horseradish peroxidase as described. Lanes 1 and 2 show the results using 2A₃ and control antibody, respectively.

β poorly when it is associated with N_{sa} (Fig. 1, lane 4) while clearly recognizing free β (Fig. 1, lane 6). The Mab also weakly recognized a peptide with an apparent M_r of 52000, the nature of which is unclear at present. Similar results were obtained with 5G₁₂ in that it clearly recognizes free β but only weakly recognized β when it associated with N_{sa} (results not shown). As expected, preincubation of either 2A₃ and 5G₁₂ with excess, unlabelled β decreases the immunoprecipitation of iodinated β .

In a further attempt to validate the specificity of the Mabs, β was transferred to nitrocellulose filter paper and treated as described in the previous section. The results in Fig. 2 indicate that 2A₃ (and 5G₁₂; results not shown) recognizes β which, under these conditions, appears to consist of two closely associated peptides, both of which are recognized by the Mabs.

Transducin is a guanine-nucleotide regulatory binding protein present in retina that mediates the activation of a cyclic GMP-dependent phosphodiesterase by light-activated rhodopsin (Stryer *et al.*, 1981). Transducin has

Fig. 3. Effect of Mab 2A₃ or control serum on the deactivation of pre-activated N_s by added β

N_s (at 8.3 μ g/ml) was activated with 20 μ M-AlCl₃/10 mM-MgCl₂/10 mM-NaF (AMF) in 50 mM-sodium Hepes, pH 8.0, containing 1 mM-EDTA, 1 mM-dithiothreitol and 0.2% Lubrol 12A9 (buffer A) for 30 min at 30 °C. The time course of deactivation of N_s was determined after a 10-fold dilution of activated N_s into 50 mM-sodium Hepes, pH 8.0, containing 5 mM-EDTA, 1 mM-dithiothreitol and 0.2% Lubrol 12A9 (buffer B) containing: no additions (○), β (3.9 μ g/ml) (△), control mouse serum (at 1 mg/ml) plus β (3.9 μ g/ml) (●), the IgG fraction of 2A₃ (1 mg/ml) (□), and 2A₃ (1 mg/ml) plus β (3.9 μ g/ml) (■), at room temperature. At the indicated times duplicate 15 μ l aliquots were removed to assay the residual N_s activity as described by Northup *et al.* (1983a). The IgG fraction of antibodies was preincubated with β for 90 min at 4 °C prior to the addition of activated N_s. Cyc⁻ membranes were generously provided by Dr. Richard B. Clark.

been purified (Fung, 1983a,b) and shown to consist of three subunits with apparent M_r values of 39000 (T _{α}), 35000 (T _{β}) and 10000 (T _{γ}). The heterotrimer appears to be structurally homologous to N_s and N_i while T _{β} is identical to N_{s β} and N_{i β} (Manning & Gilman, 1983). The cross reactivity of the Mabs against T _{β} was examined by immunoprecipitating T _{β} ; the results are shown in Fig. 1. The Mabs, not unexpectedly, immunoprecipitate T _{β} but not T _{α} (results not shown).

The results obtained to this point indicated that the Mabs recognized the β -subunit of N_s, N_i and transducin. Our next step was to determine if the Mabs could functionally modify N_s activity. Addition of the Mabs to S49 wild type or canine striatal membranes under a variety of conditions did not affect hormone- or NaF-stimulated adenylate cyclase activity (results not shown). Recently, Northup *et al.* (1983a) developed an assay to measure the activity of β which is based on the ability of the peptide to increase the rate of deactivation of preactivated N_s. We used the Mabs to see if they could functionally inhibit β activity; the results are shown in Fig. 3. Addition of β increases the rate of deactivation of N_s (Fig. 3, △) and this can be reversed by 2A₃ (Fig. 3, ■) but not by control sera (Fig. 3, ●). N_s activity in the

Fig. 4. Titration curves of Mab 5G₁₂ or control ascites fluid on the deactivation of pre-activated N_s by increasing amounts of added β

N_s was activated as described in the legend to Fig. 3. After 30 min at 30 °C, activated N_s was diluted 10-fold into buffer B containing control ascites fluid ($\square$, $\circ$, $\triangle$) or the IgG fraction of 5G₁₂ ($\blacksquare$, $\bullet$, $\blacktriangle$) at 0.1 ($\square$, $\blacksquare$), 1.0 ($\circ$, $\bullet$) or 2 ($\triangle$, $\blacktriangle$) mg/ml in the presence of various amounts of β at room temperature. At 10 min, duplicate 15 μ l aliquots were removed to assay the residual N_s activity as described in Fig. 3. The IgG fraction of the antibodies were preincubated with β at 4 °C for 90 min prior to the addition of activated N_s.

absence of β is stabilized by 2A₃ (Fig. 3, $\square$). Increasing concentrations of β decrease the activity of preactivated N_s (Fig. 4) and this can be reversed by increasing amounts of 5G₁₂ (Fig. 4, $\blacksquare$, $\bullet$, $\blacktriangle$) but not by control antibody (Fig. 4, $\square$, $\circ$, $\triangle$). Similar results are obtained if Protein A-Sepharose-purified Mabs, instead of the IgG fraction as was the case in Figs. 3 and 4, are used to inhibit β activity (Fig. 5).

DISCUSSION

In the present paper we describe the development of Mabs to the guanine nucleotide regulatory proteins associated with adenylate cyclase as well as to transducin. These proteins are functionally homologous in that they couple membrane-bound receptors to effective enzymes. The Mabs, which are of the IgG_{2b} subclass, recognize purified N_s when it is attached to a microtitre plate but only poorly recognize iodinated N_s. One reason for this difference may be that the process of fixing N_s onto the plate results in the exposure of antigenic site(s) recognized by the Mabs. Another possible reason may be that the epitope(s) on β recognized by the Mabs may be masked when β is associated with α . A third, and probably least likely, reason may be that the iodination procedure may itself cause N_s to become less antigenic, either because of

Fig. 5. Effect of purified Mabs (2A₃ or 5G₁₂) or control γ -globulin on the deactivation of N_s by added β

Clones 2A₃ and 5G₁₂ were injected into pristane-treated mice as described in the Materials and methods section. Ascites fluid was obtained and immunoglobulins were purified by using Protein A-Sepharose (Oi & Herzenberg, 1980). Commercial ascites fluid was treated in a similar manner. N_s was activated in buffer A and, after 30 min at 30 °C, diluted 10-fold in buffer B (at room temperature), containing 7.7 μ g of β /ml plus either 60 or 120 μ g of 2A₃, 5G₁₂ or control antibody/ml. After 10 min, duplicate 10 μ l aliquots were assayed for residual N_s activity. β was preincubated for 90 min at 4 °C with antibodies.

occlusion by the iodine or else some other conformational change.

N_s and N_i consist of three subunits, α , β and γ , with apparent M_r values of 42000–45000 (N_{sa}), 39000–42000 (N_{ia}), 35000 (β) and 10000 (γ). The Mabs recognize the β , but not α or γ , subunits of N_s, N_i and transducin, suggesting that these peptides are immunologically similar. These results are consistent with those of Manning & Gilman (1983) who demonstrated that the β -subunits of all three regulatory proteins were virtually identical by peptide mapping of proteolytic fragments. It is interesting that the Mabs recognize two closely associated peptides of M_r 35000, one or both of which may be the active β -subunit. We (unpublished work) and others (Sternweis & Robishaw, 1984) have noticed that, during purification of N_s, β appears to consist of two peptides. The significance of these results is unknown at present.

The available evidence suggests that receptor-mediated stimulation and inhibition of adenylate cyclase occurs when the subunits of N_s and N_i dissociate (Northup *et al.*, 1982, 1983a,b; Katada *et al.*, 1984a,b,c). N_{sa} is

believed to be the active subunit that interacts with C which leads to stimulation of adenylate cyclase (Smigel *et al.*, 1984). Inhibition of adenylate cyclase is believed to occur when the dissociated β -subunits of both N_s and N_i interact with N_{sa} to reform the holoenzyme (Northup *et al.*, 1983a,b; Katada *et al.*, 1984a,b,c). Although the role of N_{ia} in this scheme is not yet clear, a direct interaction of N_{ia} with C cannot be ruled out. The data obtained in this study support the dissociation model presented above. Using the Mabs as specific probes we have been able to neutralize β activity. The results suggest that the Mabs interact specifically with β , thereby allowing N_{sa} to interact with C. Although the precise epitope(s) for the two Mabs on β are not known, the Mabs could decrease β activity by recognizing the region of β that interacts with N_{sa} . Alternatively, the Mabs may bind β at a site unrelated to the site that associates with N_{sa} and prevent the formation of the holoenzyme entirely by steric hindrance.

Gierschik *et al.* (1985) have reported the development of polyclonal antibodies to transducins which cross-react with the β -subunits of N_s and N_i . Although these findings are similar to the results presented here, it is not known if these polyclonal antisera functionally inhibit β activity. Yatsunami *et al.* (1985), Yatsunami & Khorana (1985) and Medynski *et al.* (1985), independently of each other, recently described the development of polyclonal antisera to the subunits of transducin. Both groups used these antibodies to screen expression vector cDNA libraries for T_α . The results obtained by both groups are virtually identical with those of Tanabe *et al.* (1985) who used a mixture of monoclonal antibodies against transducin to screen a cDNA library from bovine retina. These findings, taken together with the results of the present study, suggest that monoclonal and polyclonal antibodies to the various guanine nucleotide regulatory proteins will be useful tools with which to probe the structure and function of the regulatory proteins as well as adding to our understanding of how guanine nucleotide regulatory proteins modulate the activity of the effector enzyme systems to which they are coupled.

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