# Protein Kinase Activity Associated with $Fc\gamma_{2a}$ Receptor of a Murine Macrophage Like Cell Line, $P388D_1$†

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*Received May 6, 1987; Revised Manuscript Received August 24, 1987*

ABSTRACT: The properties of protein kinase activity associated with Fc receptor specific for $IgG_{2a}$ ($Fc\gamma_{2a}R$) of a murine macrophage like cell line, $P388D_1$, were investigated. $IgG_{2a}$-binding protein isolated from the detergent lysate of $P388D_1$ cells by affinity chromatography on IgG–Sepharose was found to contain four distinct proteins of $M_r$ 50000, 43000, 37000, and 17000, which could be autophosphorylated upon incubation with [$\gamma$-$^{32}$P]ATP. The autophosphorylation of $Fc\gamma_{2a}$ receptor complex ceased when exogenous phosphate acceptors (casein or histone) were added in the reaction mixture. Casein was found to be a much better phosphate acceptor than histone in this system, as casein incorporated about 32-fold more $^{32}P$ than histone did. Phosphorylation of casein catalyzed by $Fc\gamma_{2a}$ receptor complex was dependent on casein concentration (maximum phosphate incorporation being at 0.5 mg/mL), increased with time or temperature, was dependent on the concentration of ATP and $Mg^{2+}$, and was maximum at pH near 8. Casein phosphorylation was significantly inhibited by a high concentration of $Mn^{2+}$ (>25 mM) or KCl (>100 mM) or by a small amount of heparin (>10 units/mL) and was enhanced about 2-fold by protamine. Casein kinase activity associated with $Fc\gamma_{2a}$ receptor used ATP as substrate with an apparent $K_m$ of 2 $\mu$M as well as GTP with an apparent $K_m$ of 10 $\mu$M. Prior heating (60 °C for 15 min) or treatment with protease (trypsin or Pronase) of $Fc\gamma_{2a}$ receptor complex almost totally abolished casein kinase activity. Thin-layer chromatography of a partial acid hydrolysate of the phosphorylated casein showed that the site of phosphorylation is at a seryl residue. Cyclic AMP as well as dioleine and phosphatidylserine did not increase the phosphorylation of casein catalyzed by $Fc\gamma_{2a}$ receptor complex. These results suggest that $Fc\gamma_{2a}$ receptor forms a molecule complex with protein kinase, whose characteristics resemble those of type II casein kinase but are different from those of cyclic nucleotide dependent protein kinase or from those of C protein kinase. The major casein kinase active molecule associated with $Fc\gamma_{2a}$ receptor was identified to be a protein of molecular weight near 37000 by specific labeling of $IgG_{2a}$-binding protein complex with the radioactive ATP analogue 5′-[(fluorosulfonyl)-benzoyl][$^{14}$C]adenosine.

Murine macrophages and macrophage like cell lines such as $P388D_1$ carry on their surface at least two biochemically distinct Fc receptors, one specific for $IgG_{2a}$ ($Fc\gamma_{2a}R$)[1] and another for $IgG_{2b}$ ($Fc\gamma_{2b}R$) (Walker, 1976; Heusser et al., 1977; Unkeless, 1977; Anderson & Grey, 1978; Suzuki et al., 1982). $Fc\gamma R$ plays an essential role in the antibody-dependent cell-mediated cytotoxicity (Perlman et al., 1972), the suppression of humoral immune response (Uhr & Möller, 1968) or B cell differentiation (Kölsch et al., 1980) by circulating immune complexes, or the triggering of prostaglandin synthesis by macrophages (Passwell et al., 1979, 1980; Bonn et al., 1979). Two types of $Fc\gamma R$ present on the surface of macrophages then could transmit, upon binding of specific ligands, signals unique to each type, which trigger and maintain a complex program of biochemical events, involving the generation, amplification, and propagation of a series of signals, leading to the modulation of cellular functions. A signal to trigger the arachidonic acid metabolic cascade through the cyclooxygenase pathway has been shown to be transmitted by $Fc\gamma_{2b}R$ but not by $Fc\gamma_{2a}R$ (Suzuki et al., 1982; Nitta & Suzuki, 1982a,b). Prostaglandins, which are newly synthesized by $P388D_1$ cells, then activate membrane adenylate cyclase via prostaglandin receptors (Fernandez-Botran & Suzuki, 1984a,b). $Fc\gamma_{2a}R$ was found to activate adenylate cyclase of guanine nucleotide binding stimulatory (Gs) protein/$Fc\gamma_{2a}R$ deficient cyc$^-$ cell membrane through a very different mechanism, which may involve the activation of protein kinase associated with $Fc\gamma_{2a}R$ (Fernandez-Botran & Suzuki, 1986). This paper presents the data which demonstrate that $Fc\gamma_{2a}R$ complex isolated from the detergent lysate of $P388D_1$ cells contains four distinct components which could be autophosphorylated in vitro and that $Fc\gamma_{2a}R$ complex catalyzes the phosphorylation of exogenous phosphate acceptor (casein) by enzymatic activity remarkably similar to that of type II casein kinase.

## Materials and Methods

*Cells.* The murine macrophage like cell line $P388D_1$, derived from methylcholanthrene-induced neoplasm of a DBA/2 mouse (Dawe & Potter, 1957), was a gift of Dr. H. Koren of the U.S. Environment Protection Agency at Research Triangle Park, NC. This cell line, which has been shown to possess characteristics typical for macrophages (Koren et al., 1975),

† This work was supported in part by a grant (CA 35977) from the National Cancer Institute.

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; $Fc\gamma_{2a}R$ and $Fc\gamma_{2b}R$, Fc receptors specific for $IgG_{2a}$ and $IgG_{2b}$, respectively; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; Gs protein, guanine nucleotide binding stimulatory protein; HBSS, Hank's balanced salt solution; IC, immune complex; IgG, immunoglobulin G; PAGE, polyacrylamide gel electrophoresis; PC, *rac*-1-(9-carboxynonyl)-2-hexadecylglycero-3-phosphocholine; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/87/0426-8189$01.50/0 

was grown as a monolayer in plastic flasks (Falcon 3024, Oxnard, CA) at 37 °C in an atmosphere containing 5% $CO_2$ in the culture medium consisting of RPMI 1640, heat-inactivated (56 °C, 30 min) fetal calf serum (10%), streptomycin (100 μg/mL), and penicillin (100 units/mL) (all from Hazelton Dutchland, Denver, PA), unless otherwise stated. Cell density was maintained between approximately $5 \times 10^5$ and $2 \times 10^6$ cells/mL. Under these culture conditions, the generation time was about 24 h.

*Biosynthetic Radiolabeling of P388D$_1$ Cells.* Cells ($5 \times 10^8$) were biosynthetically radiolabeled with [$^3$H]leucine (0.33 μCi/mL, Schwarz/Mann, Spring Valley, NY) for 16 h in the RPMI 1640 culture medium from which leucine was deleted, as described (Fernandez-Botran & Suzuki, 1985, 1986).

*Preparation of Lysate.* Metabolically radiolabeled cells were washed 3 times with cold Hank's balanced salt solution without $Ca^{2+}$ and $Mg^{2+}$ (HBSS; Flow Lab, Rockville, MD) and were lysed at 0 °C with 0.5% Triton X-100, made in Tris-HCl buffer (0.15 M, pH 8), containing 1 mM each of phenylmethanesulfonyl fluoride (PMSF) and ethylenediaminetetraacetate (EDTA) (buffer I). After being stirred for 1 h at 0 °C, the lysate was centrifuged for 60 min at 4 °C at 10 000 rpm in a Beckman J21 refrigerated centrifuge to remove nuclei, unlysed cells, and other debris. The clear supernatant obtained was immediately subjected to affinity chromatography.

*Affinity Chromatography and Isolation of IgG- and PC-Binding Proteins.* PC–Sepharose 4B used to extract PC-binding proteins was prepared by coupling *rac*-1-(9-carboxynonyl)-2-hexadecylglycero-3-phosphocholine (Calbiochem, LaJolla, CA) to AH-Sepharose 4B (Pharmacia, Uppsala, Sweden) in the presence of carbodiimide (Rock & Snyder, 1975), as described in detail (Suzuki et al., 1980). Heat-aggregated IgG was coupled to CNBr-activated Sepharose 4B, as described (Suzuki et al., 1980). These were packed in glass columns connected in tandem and were equilibrated against buffer I. Isolation and purification of PC- and IgG-binding proteins were carried out, as described (Suzuki et al., 1982). Briefly, the detergent lysates of P388D$_1$ cells were immediately subjected to affinity chromatography over the columns of PC– and IgG–Sepharose 4B connected in tandem in this order. After thorough washing with buffer I, the columns were disconnected. The materials bound were separately eluted with 3 M guanidine, made in 0.2 ionic strength Tris-HCl buffer (pH 8), containing 1 mM each of PMSF and EDTA (buffer II). The eluted materials were separately dialyzed against 20 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and concentrated through a YM-10 membrane to about 1 mg/mL. The dialyzed proteins were separately incubated for 30 min at 4 °C with SM-2 resin (~0.5 g wet weight; Bio-Rad, Richmond, CA) to remove Triton X-100. The proteins were recovered by centrifugation, dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% Lubrol and 10 mM $Mg^{2+}$ (buffer III), and used for autophosphorylation studies and protein kinase assays.

*Autophosphorylation of IgG-Binding Proteins.* Aliquots of the IgG- or PC-binding protein (20 μg each/assay) which were previously dialyzed against buffer III were incubated for 30 min at 30 °C with 25 μM ATP containing 25 nM [γ-$^{32}$P]ATP (sp act. of $5 \times 10^6$ cpm/nmol), in a final volume of 100 μL of buffer III. At the end of the incubation period, the reaction was stopped by being heated for 5 min at 100 °C after the addition of 50 μL of SDS–PAGE sample buffer to the samples. The samples were electrophoresed in a 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS) in the presence of 2-mercaptoethanol according to the method described by Laemmli (1970). Following the completion of the electrophoresis, the gel was stained with Coomassie blue, destained, washed, and dried for autoradiography. The extent of incorporation of $^{32}$P was examined by densitometric scan of the autoradiograph as well as by counting the radioactivity within the radioactive bands.

*Protein Kinase Assay.* Protein kinase activity potentially associated with IgG- or PC-binding proteins was assayed with the use of histone or casein as phosphate acceptors as follows. IgG- or PC-binding proteins (20 μg/assay) that were previously dialyzed against buffer III were incubated for 30 min at 30 °C with calf thymus histone (type II-AS, Sigma) or bovine casein (partially dephosphoryalted, Sigma) (5–500 μg/assay) and 25 μM ATP containing 25 nM [γ-$^{32}$P]ATP [sp act. of (1–5) $\times 10^6$ cpm/nmol] in the final volume of 100 μL of buffer III. In some experiments to examine if GTP can serve as phosphate donor, unlabeled and labeled ATP in the above assay mixture were replaced with GTP and [γ-$^{32}$P]GTP. At the end of the incubation period, the reaction was stopped by the addition of 50 μL of the SDS–PAGE sample buffer and boiling for 5 min. The samples were then electrophoresed in a 10% polyacrylamide gel in the presence of SDS as described above. The gel was then stained with Coomassie blue, destained, washed, dried, and autoradiographed as described above. The extent of $^{32}$P incorporation was examined by the densitometric scan of the autoradiograph or by counting the radioactive bands as in the case of autophosphorylation studies. Cyclic AMP dependent protein kinase assay was carried out similarly except that the assay mixture contained 1–1000 μM cyclic AMP. Protein kinase C assay was performed by including diolein (0.2 μg) (Sigma), phosphatidylserine (10 μg) (Sigma), and $Ca^{2+}$ (120 nmol) in the above assay mixture.

*In Vivo Phosphorylation.* P388D$_1$ cells ($1 \times 10^8$ per a group) were cultured with [$^{32}$P]orthophosphate (10 μCi/mL, ICN, Irvine, CA) for 30 min in the RPMI 1640 culture medium from which phosphate was deleted. Cells were then washed 3 times with the medium and cultured for additional 60 min with or without heat-aggregated murine myloma IgG$_{2a}$ or IgG$_{2b}$ (10 μg/mL). At the end of the incubation period, cells in each group were lysed with 500 μL of buffer I. The clear lysates obtained by the centrifugation were separately incubated for 6 h at 4 °C with 500 μL of protein A–Sepharose 4B (Pharmacia, Uppsala) with stirring. The materials bound to protein A–Sepharose 4B were eluted with buffer II (300 μL), after thorough washing of the gel with buffer I, and subjected to SDS–PAGE analysis as described above.

*Phospho Amino Acid Analysis.* Casein (500 μg) was incubated for 30 min at 30 °C with [γ-$^{32}$P]ATP in the presence of IgG$_{2a}$-binding protein (50 μg) in buffer III, as in the standard protein kinase assay. At the end of the incubation, the sample was dialyzed against deionized water and lyophylized. It was then hydrolyzed for 2 h at 110 °C in constant-boiling HCl (1 mL) in an evacuated sealed Pyrex tube. The hydrolysate was dried under vacuum and redissolved in 25 μL of thin-layer chromatography solvent. An aliquot (5 μL) was mixed with standard phosphoserine, -threonine, and -tyrosine (Sigma) and subjected to ascending thin-layer chromatography on a silica gel coated plate with the use of a solvent system consisting of *n*-butyl alcohol/isopropyl alcohol/formic acid/water (3:1:1:1 v/v) (Ushiro & Cohen, 1980). Following the completion of chromatography, the plate was dried, stained with 0.5% ninhydrin to locate the standard phospho amino acids, and autoradiographed with the use of X-ray film.

*Affinity Labeling with 5′-[p-(Fluorosulfonyl)benzoyl][8-$^{14}$C]adenosine.* Aliquots containing 10 μM 5′-[p-(fluorosulfonyl)benzoyl][8-$^{14}$C]adenosine (FSBA, New England Nuclear, 50 mCi/mmol) in 95% ethanol were dried by a stream of nitrogen, redissolved in 10 μL of water, and added to IgG-binding protein (20 μg in 100 μL of buffer III per experiment) in the absence or presence of a 100 molar excess of ATP, unlabeled FSBA, or 5′-adenylylimidodiphosphate. The reaction mixtures were incubated for 60 min at 22 °C. The reaction was then stopped by the addition of SDS–PAGE sample buffer (50 μL). The samples were then subjected to SDS–PAGE under reducing conditions. Upon completion of the electrophoresis, the gel was processed for autoradiography as described above.

*Other Methods.* Protein concentration was estimated by Lowry's method (Lowry et al., 1951).

## Results

Our previous studies (Fernandez-Botran & Suzuki, 1986) showed that $IgG_{2a}$-binding protein (denoted as IgG-B1) copurifies with the second protein component (denoted as IgG-B2) during the affinity chromatography of the detergent lysate of P388$D_1$ cells over a column of IgG–Sepharose. Simultaneous fusion of liposomes containing IgG-B1 and -B2 proteins with Gs protein/Fc$\gamma_{2a}$R deficient cyc$^-$ cells resulted in the formation of the hybrid membrane whose adenylate cyclase responded to immune complex formed with $IgG_{2a}$ class antibody ($IC_{2a}$) by about a 2.7-fold increase over the control. Since the activating effect of IgG-B1 and -B2 proteins could be observed in the presence of inhibitors of cAMP phosphodiesterase or of guanine nucleotide binding inhibitory protein, IgG-binding proteins appear to confer their activating effect directly on the catalytic subunit of cyc$^-$ adenylate cyclase. Since Sugden et al. (1985) recently suggested that the activation of protein kinase C may be involved in the $\alpha_1$-adrenergic potentiation of the β-adrenergic-sensitive adenylate cyclase of pineal gland and since IgG-binding protein mediated activation of cyc$^-$ adenylate cyclase was found to be inhibited by trifluoperazine, a known inhibitor of various enzymes including protein kinases (Wülfroth & Petzeit, 1985), a possible association of protein kinase activity with IgG-binding protein was examined as follows.

*Autophosphorylation of IgG-Binding Protein.* IgG- and PC-binding proteins were isolated from the detergent lysate of P388$D_1$ cells ($2 \times 10^9$) by affinity chromatography as described under Materials and Methods. They were concentrated through a YM-10 membrane to about 1 mg/mL and were dialyzed against buffer III. Aliquots of each protein (20 μg/assay) were incubated at 30 °C for 0–60 min with 25 μM ATP containing 25 nM [γ-$^{32}$P]ATP (about $1.3 \times 10^7$ cpm) in a final volume of 100 μL of buffer III. The reaction was stopped by being boiled for 5 min after the addition of 50 μL of SDS–PAGE sample buffer to each tube. The samples were then subjected to SDS–PAGE in a 10% polyacrylamide gel under reducing conditions. As illustrated by Figure 1, the autoradiograph of the SDS–PAGE gel revealed that a minimum of four proteins present in IgG-binding protein incorporated $^{32}$P, whereas PC-binding protein used as a control did not. Molecular weights of the phosphorylated IgG-binding proteins were 50 000, 43 000, 37 000, and 17 000. The extent of phosphorylation increased with time. The densitometric scan of the autoradiograph clearly showed that the protein of $M_r$ 50 000 is the one which is most phosphorylated (>50% of the total $^{32}$P incorporated).

*Phosphorylation of Casein Catalyzed by IgG-Binding Protein.* The above results suggested the association of protein kinase activity with IgG-binding protein isolated from P388$D_1$ cell lysate but not with PC-binding protein isolated from the same lysate. In order to further characterize the nature of the protein kinase activity associated with IgG-binding protein, the question of whether or not IgG-binding protein can catalyze phosphorylation of exogenous phosphate acceptors such as histone or casein was investigated next. Preliminary studies to examine which protein would be preferentially phosphorylated were carried out by incubating varying amounts (5–500 μg/assay) of histone or casein with IgG- or PC-binding proteins (20 μg/assay) and [γ-$^{32}$P]ATP in buffer III for 30 min at 30 °C. The reaction was stopped by boiling the mixtures for 5 min after addition of SDS–PAGE sample buffer. The samples were analyzed by SDS–PAGE as described under Materials and Methods. The results showed that the addition of exogenous phosphate acceptors, either histone or casein, stopped the occurrence of the autophosphorylation of IgG-binding protein. Instead, histone or casein was found to be phosphorylated. The extent of $^{32}$P incorporation into casein was about 32-fold more than that into histone (data not shown). And the phosphorylation of casein increased with its concentration, reaching a maximum when 50 μg of casein was used per assay. Further increase in casein concentration decreased the incorporation of $^{32}$P into casein (data not shown). Therefore, the characterization of protein kinase activity associated with IgG-binding protein was performed with the use of casein as phosphate acceptor at 50 μg per assay as follows.

*Factors Affecting Protein Kinase Activity Associated with IgG-Binding Protein.* Various factors that may affect the protein kinase activity were investigated by incubating casein (50 μg/assay) with IgG-binding protein (20 μg/assay) under various conditions for 30 min at 30 °C. In each experiment, the reaction was stopped by boiling the samples for 5 min after the addition of SDS–PAGE sample buffer. The reaction mixtures were subjected to SDS–PAGE and analyzed as de-

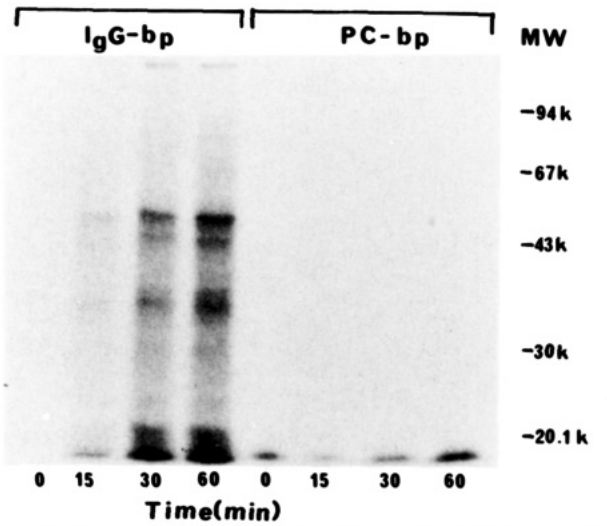


FIGURE 1: Progression of autophosphorylation of IgG-binding protein complex with time. IgG-binding protein (IgG-bp) and PC-binding protein (PC-bp) were isolated from the detergent lysate of P388$D_1$ cells ($2 \times 10^9$) by affinity chromatography as described under Materials and Methods. They were separately concentrated to about 1 mg/mL and dialyzed against buffer III. Aliquots of each protein (20 μg/assay) were incubated at 30 °C for 0–60 min with 25 μM ATP containing 25 nM [γ-$^{32}$P]ATP (about $1.3 \times 10^7$ cpm) in final volume of 100 μL of buffer III. The reaction was stopped by being boiled for 5 min after the addition of 50 μL of SDS–PAGE sample buffer to each tube. The samples were electrophoresed in a 10% polyacrylamide gel in the presence of SDS under reducing conditions. the gel was processed for autoradiography as described under Materials and Methods.

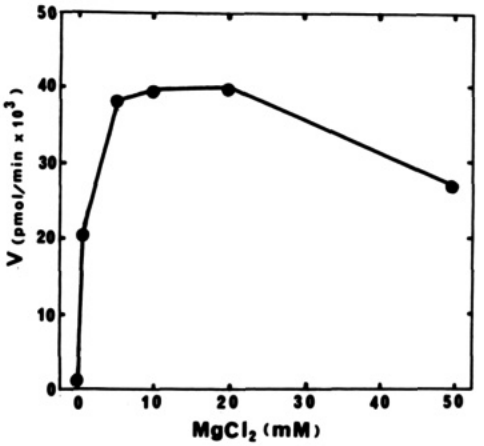


FIGURE 2: Effects of $Mg^{2+}$ concentration on casein kinase activity associated with IgG-binding protein. Casein (50 $\mu$g/assay) was incubated at 30 °C at pH 8 for 30 min with IgG-binding protein (20 $\mu$g/assay) and 25 $\mu$M ATP containing 25 nM [$\gamma$-$^{32}$P]ATP in 50 mM Tris-HCl buffer containing 0.1% Lubrol and various concentration of $Mg^{2+}$ (0–50 mM). The degree of $^{32}$P incorporation into mixtures was estimated by counting the radioactive bands of the dried SDS–PAGE gel as described under Materials and Methods.

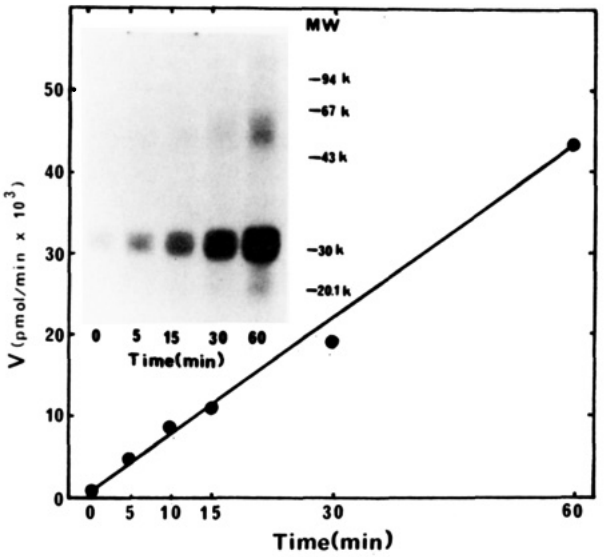


FIGURE 3: Time course of phosphorylation of casein catalyzed by IgG-binding protein. Casein kinase activity of IgG-binding protein was assayed as in Figure 2, except that the time of incubation was varied from 0 to 60 min. The insert is the autoradiography of the SDS–PAGE gel of the assay mixtures incubated for 0, 5, 15, 30, and 60 min.

scribed under Materials and Methods. First, the optimum pH for phosphorylation of casein catalyzed by IgG-binding protein was found to be between pH 6 and pH 8. At pH below 5.5, essentially no phosphorylation of casein was observed. At alkaline pH above 8.5, the rate of $^{32}$P incorporation into casein was reduced to about 60–70% of that at pH 8. As shown by Figure 2, phosphorylation of casein at pH 8 required the presence of $Mg^{2+}$, as no phosphorylation proceeded in the absence of $Mg^{2+}$. The addition of 5–20 mM $Mg^{2+}$ in the assay system promoted the rate of $^{32}$P incorporation to about 0.04 pmol/min, whereas the addition of 50 mM reduced the rate to about 0.027 pmol/min. At pH 8 and in the presence of 10 mM $Mg^{2+}$, the addition of $Mn^{2+}$ up to 2.5 mM had very little effect on IgG-binding protein catalyzed phosphorylation of casein. However, the increase of $Mn^{2+}$ concentration to 25 mM resulted in the inhibition of the rate of $^{32}$P incorporation from about 0.038 to 0.009 pmol/min. And the addition of an increasing concentration of KCl (>50 mM) progressively inhibited the phosphorylation of casein (from about 0.06 pmol/min in the absence of KCl to 0.009 pmol/min in the presence of 0.3 M KCl). Figure 3 illustrates that at pH 8, in the presence of 10 mM $Mg^{2+}$, and in the absence of KCl and $Mn^{2+}$, the phosphorylation of casein catalyzed by IgG-binding protein increased with time almost linearly up to 60 min.

Casein kinase activity associated with IgG-binding protein increased with the function of the ATP concentration and appeared to plateau at above 10 nM. The double-reciprocal plot of enzyme kinetics gave an apparent $K_m$ value of 2 $\mu$M, suggesting a relatively strong affinity of casein kinase associated with IgG-binding protein for ATP. In order to investigate if casein kinase activity associated with IgG-binding protein can utilize GTP as phosphate donor, casein kinase activity was assayed in the reaction system in which ATP was replaced with [$\gamma$-$^{32}$P]GTP as phosphate donor, as described under Materials and Methods. Results showed a clear incorporation of $^{32}$P into casein (data not shown). However, the apparent $K_m$ value estimated by the double-reciprocal plot of enzyme kinetics was about 10 $\mu$M, suggesting that the affinity of casein kinase associated with IgG-binding protein for GTP is considerably weaker than that for ATP.

*Effects of Heparin or Protamine on Casein Kinase Activity Associated with IgG-Binding Protein.* The above results strongly suggested that the properties of the protein kinase activity associated with IgG-binding protein resemble those of casein kinase II in their pH optimum, requirement for $Mg^{2+}$, and $K_m$ values for ATP and GTP. Since casein kinase II has been shown to be inhibited specifically by heparin (Hathaway et al., 1980) and enhanced by polyamines such as protamine (Cochet et al., 1980), the effects of heparin (Sigma) and protamine (Sigma) on casein kinase activity associated with IgG-binding protein were next investigated. The addition of heparin into the assay system at a concentration greater than 10 units/mL resulted in the inhibition (>75%) of the phosphorylation of casein catalyzed by IgG-binding protein. The addition of protamine at 0.1 mg/mL in the standard assay mixture resulted in about a twofold increase of IgG-binding protein catalyzed phosphorylation of casein.

*Other Factors Affecting Protein Kinase Activity Associated with IgG-Binding Protein.* Addition of cyclic AMP (1–1000 $\mu$M) or of protein kinase C activator, diolein (0.2 $\mu$g), phosphatidylserine (10 $\mu$g), and $Ca^{2+}$ (120 nmol) to the standard assay system was found to cause no increase in $^{32}$P incorporation into casein. Addition of cAMP (1 mM) rather suppressed the $^{32}$P incorporation into casein from the control level of 0.025 to 0.004 pmol/min. Protein kinase C activators also slightly inhibited (<10%) the casein kinase II activity of IgG-binding protein. Prior treatment of IgG-binding protein with heat (60 °C, for 15 min) reduced the rate of $^{32}$P incorporation into casein by 90%. The prior treatment with Pronase or trypsin of IgG-binding protein (enzyme:substrate ratio of 1:100) totally abolished its catalytic activity (>95%).

*Phosphorylation Site.* In order to investigate which amino acid residues were modified during IgG-binding protein catalyzed phosphorylation, casein that was incubated with IgG-binding protein in the presence of [$\gamma$-$^{32}$P]ATP was hydrolyzed for 2 h at 110 °C with constant-boiling HCl in an evacuated and sealed Pyrex tube. The hydrolysate was dried under vacuum and reconstituted in 25 $\mu$L of the thin-layer chromatography solvent described below. An aliquot (5 $\mu$L) was spotted on a silica gel plate and subjected to ascending thin-layer chromatography with the use of a solvent system consisting of *n*-butyl alcohol/isopropyl alcohol/formic acid/water (3:1:1:1 v/v). The autoradiography of the thin-layer gel clearly

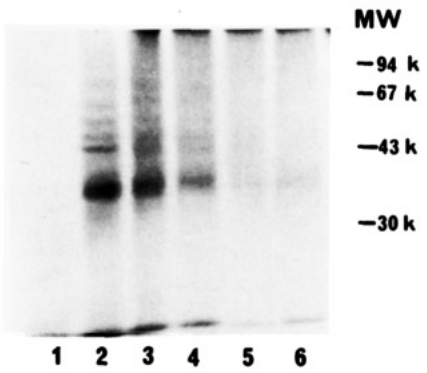


FIGURE 4: Affinity labeling of IgG-binding protein with 5′-[*p*-(fluorosulfonyl)benzoyl][$^{14}$C]adenosine (FSBA). IgG-binding protein (20 $\mu$g) (lane 2) or PC-binding protein (20 $\mu$g) (lane 1) in buffer III was incubated at 22 °C for 60 min with 10 $\mu$M [$^{14}$C]FSBA in the absence (lanes 1 and 2) or presence of a 100 molar excess of unlabeled ATP (lane 4), FSBA (lane 5), or 5′-adenylyl imidodiphosphate (lane 6). Lane 3 contains IgG-binding protein that was incubated with [$^{14}$C]FSBA after being heated for 15 min at 60 °C. Following the incubation, the samples were subjected to SDS–PAGE under reducing conditions. The gel was then processed for autoradiography.

demonstrated that casein kinase activity associated with IgG-binding protein modified only seryl residues.

*Identification of Protein Kinase Active Molecule Associated with IgG-Binding Protein.* The results presented above thus suggested that the Fc$\gamma_{2a}$R isolated as IgG-binding protein is associated with protein kinase activity that is capable of the catalysis of phosphorylation of exogenous substrate, particularly the acidic protein casein. In order to identify the molecule responsible for this activity, IgG-binding protein was incubated at 22 °C for 60 min with 10 $\mu$M 5′-[*p*-(fluorosulfonyl)-benzoyl][$^{14}$C]adenosine (FSBA), an ATP analogue known to modify ATP-binding sites (Miller-Diener et al., 1985; Woodford & Pardee, 1986), in the absence or presence of a 100 molar excess of ulabeled ATP, FSBA, or 5′-adenylyl-imidodiphosphate. The samples were subjected to SDS–PAGE under reducing conditions. Upon completion of electrophoresis, the gel was processed for autoradiography. Figure 4 illustrates the result obtained. The incubation of IgG-binding protein with [$^{14}$C]FSBA in the absence of other substances resulted in radiolabeling of one major band of molecular weight near 35 000–37 000 and several minor bands of molecular weight greater than 43 000 (lane 2). PC-binding protein which was shown to possess no detectable protein kinase activity in this study did not contain any ATP-binding protein (lane 1). The prior heating of IgG-binding protein at 60 °C for 15 min, which reduced kinase activity by 90%, did not appear to affect the binding of [$^{14}$C]FSBA to the protein of molecular weight near 37 000 (lane 3). The presence of 1 mM ATP (lane 4) inhibited the radiolabeling of $M_r$ 35 000–37 000 protein by about 90%. The presence of 1 mM unlabeled FSBA or 5′-adenylyl imidodiphosphate during the incubation with [$^{14}$C]FSBA almost totally abolished the radiolabeling of $M_r$ 35 000–37 000 protein (lanes 5 and 6, respectively), suggesting the [$^{14}$C]FSBA indeed modified the ATP-binding site of this protein.

*In Vivo Phosphorylation.* Although the data presented above suggested a probable association of casein kinase activity with $IgG_{2a}$-binding protein isolated from the detergent lysate of $P388D_1$ cells, a question was raised whether or not the binding of $IgG_{2a}$ to cell surface Fc$\gamma_{2a}$R leads to the autophosphorylation of Fc$\gamma_{2a}$R. This question was investigated as follows. $P388D_1$ cells ($3 \times 10^8$) were cultured for 30 min with [$^{32}$P]orthophosphate (10 $\mu$Ci/mL) in the RPMI 1640 culture medium from which phosphate was deleted. They were

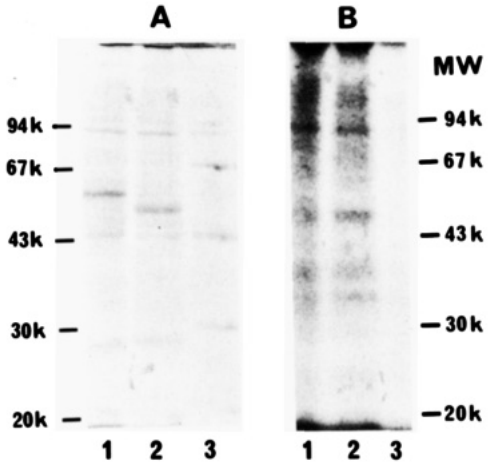


FIGURE 5: In vivo phosphorylation of $IgG_{2a}$-binding protein. $P388D_1$ cells ($1 \times 10^8$ per group) that were prelabeled with [$^{32}$P]orthophosphate as described under Materials and Methods were incubated for 60 min without (lane 3) or with heat-aggregated murine myeloma $IgG_{2a}$ (lane 2) or $IgG_{2b}$ (lane 1) (both at 10 $\mu$g/mL). Cells in each group were then lysed with 500 $\mu$L of buffer I. The clear supernatants obtained by centrifugation of each lysate were incubated for 6 h with stirring at 4 °C with protein A–Sepharose 4B (500 $\mu$L). Following the incubation period, the materials bound to protein A–Sepharose 4B were eluted with buffer II and subjected to SDS–PAGE under reducing conditions. (A) Coomassie blue stained pattern; (B) autoradiograph.

then washed with the culture medium to remove excess free $^{32}$P, divided into three groups, and cultured for 60 min with or without heat-aggregated $IgG_{2a}$ or $IgG_{2b}$ (10 $\mu$g/mL). At the end of the culture period, cells in each group were collected, washed with PBS, and lysed with 500 $\mu$L of buffer I. Following the centrifugation of the lysates, the clear supernatants were incubated with stirring at 4 °C for 6 h with protein A–Sepharose 4B (500 $\mu$L of settled gel). After the incubation period, protein A–Sepharose 4B was recovered from the mixtures by centrifugation and washed 3 times with cold buffer I. The materials bound to the gel were eluted with 200 $\mu$L of buffer II and subjected to SDS–PAGE. As shown by Figure 5, the stimulation of $P388D_1$ cells with $IgG_{2a}$ appeared to lead to the phosphorylation of the protein of molecular weight near 50 000, which was absent in the detergent lysate of the cells stimulated with $IgG_{2b}$ or of the unstimulated cells.

## Discussion

The rationale behind the present studies is the previous findings which showed that the adenylate cyclase activity of the hybrid membrane, formed by fusion of Fc$\gamma_{2a}$R/Gs protein deficient cyc$^-$ cells with liposome containing IgG-binding protein isolated from $P388D_1$ cells, was increased in response to $IC_{2a}$, but not to $IC_{2b}$, about 2.7-fold over the control hybrid membrane between cyc$^-$ cells and liposome containing no protein (Fernandez-Botran & Suzuki, 1986). Since this activating effect of IgG-binding protein on cyc$^-$ adenylate cyclase was suppressed by trifluoperazine, an inhibitor of various enzymes including protein kinases (Wülfroth & Petzeit, 1985), and since the involvement of protein kinase C activation in $\alpha_1$ potentiation of $\beta$-adrenergic-sensitive adenylate cyclase of pineal gland has been demonstrated (Sugden et al., 1985), the activation of the catalytic subunit of adenylate cyclase by Fc$\gamma_{2a}$R complex could involve the activation and deactivation of protein kinase activity potentially associated with Fc$\gamma_{2a}$R. The data of Figure 1 suggest that $IgG_{2a}$-binding protein complex, isolated from the detergent lysate of $P388D_1$ cells by affinity chromatography on IgG–Sepharose, indeed contains protein kinase activity, because the incubation of $IgG_{2a}$-binding

protein complex with [$\gamma$-$^{32}$P]ATP led to the phosphorylation of a minimum of four proteins. We have previously shown that the protein of $M_r$ 50000 which incorporated the highest amount of $^{32}$P under the experimental condition used is Fc$\gamma_{2a}$R molecule, which has an isoelectric point lower than 4.5 and possesses the specific $IgG_{2a}$-binding capacity (Suzuki et al., 1982). The protein of $M_r$ 35000–37000, which is also autophosphorylated, could be the protein kinase active molecule, because this was the major protein that bound the radioactive ATP analogue, [$^{14}$C]FSBA (Figure 4). The blocking of [$^{14}$C]FSBA binding to this protein by ATP or 5′-adenylylimidodiphosphate suggests that this protein has a specific ATP-binding site, although a question of whether the modification by FSBA inhibits protein kinase activity associated with $IgG_{2a}$-binding protein needs to be examined. Efforts are currently being made in our laboratory to isolate the protein kinase active molecule by fractionating the Fc$\gamma_{2a}$R complex into each component protein.

The characteristics of the protein kinase activity associated with Fc$\gamma_{2a}$R remarkably resemble casein kinase II, which has been identified in both the nucleus and the cytoplasm of a wide variety of eukaryotic cells (Hathaway & Traugh, 1982). Like casein kinase II; the kinase activity associated with $IgG_{2a}$-binding protein catalyzed phosphorylation of the acidic protein casein much more efficiently than that of the basic protein histone. It can utilize GTP as substrate in addition to ATP. The apparent $K_m$ values for ATP and for GTP of 2 $\mu$M and 10 $\mu$M, respectively, are comparable to those reported for casein kinase II from various tissues ($K_m$ values for ATP reported to range from 4 to 62 $\mu$M, whereas those for GTP range from 7 to 48 $\mu$M) (Hathaway & Traugh, 1982). The inhibition and the activation of protein kinase activity associated with Fc$\gamma_{2a}$R by heparin and by protamine, respectively, are also typical properties of casein kinase II of many different tissues (Hathaway & Traugh, 1982). Heparin, which has been shown to be most potent and specific for casein kinase II (Hathaway et al., 1980), was found, from the reciprocal plot of the enzyme kinetics data, to increase the apparent $K_m$ for casein from about 6 $\mu$M in the absence of heparin to 67 $\mu$M in the presence of heparin.

Casein kinase II of various cell types has been shown to consist of two or three types of subunits to form a complex of molecular weight near 130000 (Hathaway & Traugh, 1979). The molecular weight ranging from 44000 to 35000 has been reported for the largest subunit, denoted as $\alpha$, which has been shown to have an ATP-binding site (Hathaway et al., 1982). This subunit may be degraded upon standing at 4 °C for a long period of time into $\alpha'$ subunit of molecular weight near 37000 without losing its catalytic activity (Boivin & Garland, 1979; Hathaway & Traugh, 1982). The smallest subunit, which is denoted as $\beta$, has a molecular weight ranging from 24000 to 26000. This subunit is shown to be the acceptor site for autophosphorylation (Hathaway & Traugh, 1979). The results of the specific binding of [$^{14}$C]FSBA to IgG-binding protein (Figure 4) suggest that the major protein kinase active molecule may be in $\alpha'$ form of $M_r$ of 37000, which could have been degraded during the lysis of cells and affinity chromatography from $\alpha$ form of $M_r$ of 43000, which was also radiolabeled specifically with [$^{14}$C]FSBA. Casein kinase II activity associated with $IgG_{2a}$-binding protein may not have a $\beta$ subunit, because the autophosphorylation experiment (Figure 1) did not show the presence of a radioactive band in the molecular weight range of 24000–26000. However, the autophosphorylated band of molecular weight near 17000 could be the degradation product of the $\beta$ subunit. It is also possible that the Fc$\gamma_{2a}$R molecule, the major phosphate acceptor during autophosphorylation, may be still associated with the $\beta$ subunit or contain a structure similar to the $\beta$ subunit. Another possibility is that the casein kinase activity associated with Fc$\gamma_{2a}$R may belong to type I, whose molecular weight was reported to be close to 37000, although characteristics other than molecular weight do not exactly suggest that this may be the case.

Although protein kinase activity associated with Fc$\gamma_{2a}$R remarkably resembles casein kinase II of various tissues in many properties as described above, it differs from classical casein kinase II in two aspects, in addition to the afore-mentioned difference in molecular weight of the $\alpha$ subunit and the apparent lack of a $\beta$ subunit which could be autophosphorylated. First, casein kinase from other tissues has been shown to phosphorylate preferentially threonyl residues of substrate (Hathaway & Traugh, 1979; Matsumura & Takeda, 1972; Thornburg et al., 1977, 1979), whereas protein kinase associated with Fc$\gamma_{2a}$R appeared to be specific for the seryl residue of casein. This could be due to the use of partially dephosphorylated casein in the assay, since such substrate has been shown to be preferentially phosphorylated at seryl residues by casein kinase II (Donella-Deana et al., 1979; Pinna et al., 1979). Second, the protein kinase activity associated with $IgG_{2a}$-binding protein was suppressed by the addition of KCl, whereas casein kinase II from rabbit reticulocytes apparently requires the optimal concentration of this salt (150 mM) (Hathaway & Traugh, 1983). The reason for this discrepancy is not clear at present.

Receptors for epidermal growth factor or for insulin have been shown to possess intrinsic tyrosine kinase activity and undergo autophosphorylation upon ligand binding. These receptors are also subjected to phosphorylation on seryl and threonyl residues, which is in part catalyzed by protein kinase C (Hunter & Cooper, 1985; Gammeltoft & Van Obberghen, 1986; Nishizuka, 1986). Such phosphorylation of receptors was suggested to lead to the trapping of recycling receptors within the cell, either through the acceleration of internalization or by a retardation of the movement to the cell surface (Klausner et al., 1984; Beguinot et al., 1985; Lin et al., 1986). The protein kinase associated with Fc$\gamma_{2a}$R appears to be neither tyrosine specific nor diolein–phosphatidylserine sensitive. It was found also to be not dependent on cAMP. Thus, whether or not phosphorylation of the Fc$\gamma_{2a}$R molecule by a casein kinase II like enzyme associated with Fc$\gamma_{2a}$R would lead to the trapping of this receptor within the cell may have to be examined in the future.

Casein kinase exists primarily in cytoplasm and nuclei. However, the association of casein kinase II with membrane has been demonstrated in rabbit and sheep erythrocytes, which may be involved in the phosphorylation of glycophorins (Hosey & Tao, 1977). Casein kinase II appears to be associated also with coated vesicles (Bar-Zvi & Branton, 1986) where many cell surface receptors are concentrated during endocytosis (Goldstein et al., 1979). Recently, Kishimoto et al. (1987) reported that low-density lipoprotein (LDL) receptor isolated from bovine adrenal cortex exhibits casein kinase II like activity which phosphorylates the serine at position 833 of the receptor itself. Their results showed that LDL receptor kinase shares some properties with casein kinase II, since it can use either GTP or ATP as phosphate donor and is inhibited by heparin. However, it was shown also to be different from classical casein kinase II in molecular weight (500000 compared to 130000 for casein kinase II), apparent $K_m$ for LDL receptor (5 nM compared to 10 $\mu$M for casein kinase II), and

response to polylysine (inhibition rather than activation as expected with casein kinase II). In addition, it is not clear whether kinase activity is tightly associated with LDL receptor or is a part of the LDL receptor molecule, due to its loss of activity by exposure to high salt concentration which may be needed to separate the activity.

The physiological significance of the association of casein kinase activity with Fc$\gamma_{2a}$R is still open to question. The association is probably specific, because the casein kinase activity appeared to be very tightly bound to Fc$\gamma_{2a}$R protein and because Fc$\gamma_{2b}$R isolated as PC-binding protein from the same cell lysate did not exhibit casein kinase activity and could not be radiolabeled with [$^{14}$C]FSBA. Because Fc$\gamma_{2a}$R protein is an acidic protein with an isoelectric point below 4.5, this could be a natural substrate for the casein kinase II like enzyme, which is known to prefer an acidic protein such as casein. Indeed, the data of Figure 1 suggest that more than 50% of the total $^{32}$P incorporated into four different proteins was found to be associated with a protein of $M_r$ 50 000, which has been previously shown to be the size of Fc$\gamma_{2a}$R (Suzuki et al., 1982). Furthermore, the preliminary result of in vivo phosphorylation studies (Figure 5) supports the contention that Fc$\gamma_{2a}$R at the viable cell surface may be a natural substrate for casein kinase like enzyme, because the phosphorylation of $M_r$ 50 000 protein could be observed specifically in the lysate of the cells that were stimulated with $IgG_{2a}$. But a question remains as to the relationship between the phosphorylation of Fc$\gamma_{2a}$R and the activation of the catalytic subunit of adenylate cyclase.

**Registry No.** Casein kinase, 52660-18-1.

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