Mono-ADP-ribosylation is a post-translational modification of cellular proteins that has been implicated in the regulation of signal transduction, muscle cell differentiation, protein trafficking, and secretion. In several cell systems we have observed that the major substrate of endogenous mono-ADP-ribosylation is a 36-kDa protein. This ADP-ribosylated protein was both recognized in Western blotting experiments and selectively immunoprecipitated by a G protein β subunit-specific polyclonal antibody, indicating that this protein is the G protein β subunit. The ADP-ribosylation of the β subunit was due to a plasma membrane-associated enzyme, was sensitive to treatment with hydroxylamine, and was inhibited by meta-iodobenzylguanidine, indicating that the involved enzyme is an arginine-specific mono-ADP-ribosyltransferase. By mutational analysis, the target arginine was located in position 129. The ADP-ribosylated β subunit was also deribosylated by a cytosolic hydrolase. This ADP-ribosylation/deribosylation cycle might be an in vivo modulator of the interaction of βγ with specific effectors. Indeed, we found that the ADP-ribosylated βγ subunit is unable to inhibit calmodulin-stimulated type 1 adenyl cyclase in cell membranes and that the endogenous ADP-ribosylation of the β subunit occurs in intact Chinese hamster ovary cells, where the NAD+ pool was labeled with [3H]adenine. These results show that the ADP-ribosylation of the βγ subunit could represent a novel cellular mechanism in the regulation of G protein-mediated signal transduction.

Several cell proteins can be modified by covalent reactions that affect their function. Whereas the best described modification involves the phosphorylation of specific residues, evidence is also accumulating that endogenous ADP-ribosylation can play a similar role (Ref. 1 and references therein). Enzymatic mono-ADP-ribosylation involves the transfer of the ADP-ribose moiety from NAD to a specific amino acid of cellular proteins. The best characterized mono-ADP-ribosylation reactions are those catalyzed by the bacterial toxins, such as pertussis (2), cholera (3), diphtheria (4), and clostridial toxins (5, 6). These bacterial ADP-ribosyltransferases act by modifying crucial proteins, such as the α subunit of the heterotrimeric GTP-binding proteins (G proteins)† (2, 3), the small monomeric GTP-binding protein Rho (5), monomeric actin (6), and elongation factor-2 (4), resulting in permanent activation or inactivation of critical cell functions. For example, cholera toxin, an arginine-specific ADP-ribosyltransferase, ADP-ribosylates the α subunit of the stimulatory G protein (Gs) to irreversibly inhibit its GTPase activity (3, 7, 8).

Endogenous mono-ADP-ribosylation has also been described in eukaryotic cellular systems; ADP-ribosyltransferases that catalyze ADP-ribosylation of arginine residues of G proteins (similar to cholera toxin) have been described in many cells and tissues (9–12). The endogenous ADP-ribosylation of cytosine residues of membrane G proteins (similar to pertussis toxin) has also been suggested to occur in erythrocytes (13, 14). Thus, some of these enzymes are able to modify G proteins (15–17) and presumably play a role in signal transduction, although their substrates have been poorly characterized and their functional significance is even less understood.

It has also been proposed that an ADP-ribosyltransferase may be coupled to an ADP-ribosylarginine hydrolase that is able to remove the ADP-ribose group and hence regenerate free arginine, completing an ADP-ribosylation cycle that can reversibly regulate the functions of substrate proteins (18, 19). An example of this cycle in eukaryotes is given by desmin, the muscle-specific intermediate filament protein as follows: ADP-ribosylation blocks the assembly of desmin into 10-nm filaments in vitro, and an incubation with ADP-ribosylarginine hydrolase restores the self-assembly properties of desmin (20–22).

Here we report the direct demonstration of endogenous mono-ADP-ribosylation of the G protein β subunit, and we provide evidence that this modification can modulate βγ activity in a similar way to the regulation of some G protein α subunits. Thus we propose that the ADP-ribosylation/deribosylation cycle of the βγ subunit might represent a novel cellular mechanism to regulate G protein-mediated signal transduction.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM) and Hanks’ Balanced Salt Solution (HBSS) were purchased from Life Technologies, Inc.; [32P]NAD was from Amersham Pharmacia Biotech, and [2,8-3H]adenine was from NEN Life Science Products. Cholera toxin was from Calbiochem, and pertussis toxin was a generous gift of Dr. R. Rappuoli (Chiron Vaccines, Siena, Italy). Tosylphenylalanyl chlorom-

† The abbreviations used are: G protein, GTP-binding protein; CHO cells, Chinese hamster ovary cells; DTT, dithiothreitol; NH2OH, hydroxyamine; MIBG, meta-iodobenzylguanidine; GPI, glycosylphosphatidylinositol; AC1 and AC2, adenyl cyclase types 1 (AC1) and 2 (AC2); CaM, Ca++/calmodulin; DMEM, Dulbecco’s modified Eagle’s medium; HBSS, Hanks’ Balanced Salt Solution; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)aminoethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.
ethyl ketone-treated trypsin was from Worthington. Other chemicals used were obtained from Sigma at the highest available purities. Part of the purified bovine brain \( \beta \) and the antibodies raised against the carboxyl terminus and the amino terminus of \( \beta \) subunit were generously supplied by Dr. W. F. Simonds (National Institutes of Health, Bethesda). Antibody-encoding His6-terminus (specific activity, 20–40 Ci/mmoll) in 1 ml of DMEM without fetal calf serum, containing 20 \( \mu \)g/ml actinomycin D to avoid \([\text{H}]\)adenine incorporation into RNA. The labeled medium was replaced with fresh fresh medium, and the incubation was carried out for an additional 4 h in the absence or in the presence of pertussis toxin (100 ng/ml). Then the supernatant was removed, and the incubation was continued for 10 min at 37 °C with RNase (100 \( \mu \)g/ml) in 100 \( \mu \)l of 20 mM Tris-HCl (pH 7.5). The reaction was terminated by diluting the samples with 50 \( \mu \)l of Laemmli sample buffer, followed by 2 min boiling and analysis by 10% SDS-PAGE. The protein associated radioactivity was evaluated by a Bio-Imageing Analyzer (FUJI Film) or by fluorography with the gels exposed for at least 20 and 90 days, respectively. To verify the \([\text{H}]\)adenine incorporation into the cellular \( \text{NAD}^+ \) pool, radiolabeled CHO cells were extracted with methanol/chloroform/water/12 \( \% \) HCl (1:1:0.5:0.01) and analyzed by HPLC as described above, with the following modifications: \( \text{H}_2\text{O} \) for the first 5 min, followed by a linear gradient of 0–30 \( \% \) ammonium phosphate (5–55 min), and of 30 \( \% \) to 1 \( \% \) (55–115 min). \([\text{H}]\)NAD represented ~8% of the total \([\text{H}]\)adenine metabolites. Moreover, the CHO intracellular \( \text{NAD}^+ \) concentration was determined, considering that 10\(^6\) cells have a volume of 1.2 \( \mu \)l (as measured by a Coulter Counter ZM linked to a Coulter Channelizer 256), and was found to be 785 ± 10 \( \mu \)M. This allows the calculation of the specific activity of the \( \text{NAD}^+ \) pool as 380 \( \mu \)Ci/mmol, the value used to calculate the amount of the endogenously mono-ADP-ribosylated \( \beta \) subunit.

**Affinity Purification of the Endogenously \([\text{H}]\)-Labeled \( \beta \) Subunit—** \([\text{H}]\)-Adenine-labeled CHO cells (6 \( \times \) 10\(^5\)) for each experiment) were washed with HBSS and then broken with a Teflon/glass Potter homogenizer in hypotonic buffer containing 5 mM Tris-HCl (pH 8.0), 1 mM EDTA, and protease inhibitors. Unbroken cells and nuclei were removed by low speed centrifugation (10 min at 600 \( \times \)g), and the crude membranes (300 \( \mu \)g) were collected by centrifuging the supernatant for 15 min at 25,000 \( \times \) g. The \([\text{H}]\)-labeled \( \beta \) subunit was extracted and analyzed as described previously (32), with the difference that His\(_{\alpha}\), \( \alpha_i \) was employed to reassociate with \( \beta \) \( \gamma \). After washing with NaSCN, the bound \( \beta \) \( \gamma \) subunits were solubilized in Laemmli sample buffer and analyzed as described above. About 2 \( \mu \)g of \( \beta \) \( \gamma \) were recovered from this affinity purification (as estimated by densitometric analysis of the immunoblot) with ~0.2% being modified in intact cells.

**Adenylyl Cyclase Assay—** Membranes (25 \( \mu \)g/25 \( \mu \)l) prepared from freshly dissected rat brain (33, 34) were assayed for adenylyl cyclase activity as described previously (35), with the exception that cAMP was separated by thin layer chromatography using silica gel G plates (Kieselgel 60 F\(_{254}\), Merck) pretreated with 1% potassium oxalate and 2 \( \text{mM} \) EDTA, with chloroform/methanol/4 \( \% \) \( \text{NH}_4\text{OH} \) (54:42:12) as the solvent system. The effect of the adenylyl- \( \beta \) \( \gamma \) subunit was evaluated by adding to the reaction mixture \( \mu \)g of plasma membranes from CHO cells preincubated for 6 h at 37 °C with different concentrations of purified bovine brain \( \beta \) \( \gamma \) subunit (100–500 \( \mu \)l) in the absence (“unmodified”) or presence (“modified”) of \([\text{H}]\)NAD (1 \( \mu \varepsilon \)). Under these experimental conditions about 60–80% of the added \( \beta \) \( \gamma \) subunit was ADP-ribosylated. After the incubation, CHO membranes containing either unmodified or modified \( \beta \) \( \gamma \) subunit were centrifuged (15 min at 12,000 \( \times \) g) and resuspended in 5 \( \mu \)l of 10 \( \mu \mM \text{HEPES} \) (pH 8.0) and 0.1% Lubrol, and then added to the adenylyl cyclase assay mixture.

**Other Methods—** Point mutations were generated using the Quickchange, site-directed mutagenesis kit (Stratagene), and the sequence of all constructs was confirmed by automated DNA sequencing. Snake venom phosphodiesterase digestion of ADP-ribosylated proteins (33, 36), production of \([\text{H}]\)ADP-ribose (36), sensitivity of the ADP-ribosylated protein to hydroxylamine (\( \text{NH}_2\text{OH} \)) and HgCl\(_2\) (36), and phosphonitride-specific phospholipase C assay (37) were performed as described previously.

**RESULTS AND DISCUSSION**

**Identification of a 36-kDa ADP-ribosylated Protein as the G Protein \( \beta \) Subunit—** Substrates of endogenous ADP-ribosylation can be identified by supplying cell extracts with radiolabeled \( \text{NAD}^+ \). In enriched plasma membrane preparations from different cell lines, including Swiss 3T3, CHO and HL60 cells, \( \text{NAD}^+ \) prominently labeled a 36-kDa protein (Fig. 1A). In the CHO preparation, the labeled protein co-migrated with the purified G protein \( \beta \) subunit on SDS-PAGE and was recognized on Western blots by a polyclonal antibody raised against the
carboxyl-terminal decapeptide of the β subunit (SW28, which stains a doublet representing the β1 and β2 isoforms) (Fig. 1, B and C). Purified bovine brain βγ subunit added to the assay mixture was also ADP-ribosylated (Fig. 1A, lanes 2, 4, 6, 8, and 10) and precisely co-migrated with the ADP-ribosylated 36-kDa endogenous protein on SDS-PAGE (Fig. 1A, lanes 1, 3, 5, 7, and 9 and Fig. 1B, lane 2, as lane 1 with purified bovine brain βγ subunit (2.5 μg/ml) added; lane 3, control for lane 4 (no toxin added); lane 4, pertussis toxin-dependent ADP-ribosylation of αs; lane 5, control for lane 6 (no toxin added); lane 6, cholera toxin-dependent ADP-ribosylation of αs, lanes 7 and 8 show the immunoblots of lanes 4 and 2; β1 and β2 are the two β isoforms identified by the SW28 antibody. The data shown are from a single experiment performed in duplicate, which is representative of at least three independent experiments.

The modified amino acid of the β subunit was further investigated by means of a characterization of the chemical stability of the ADP-ribose linkage. Treatment of the [32P]ADP-ribosylated β subunit for 12 h with NH2OH (which hydrolyzes the ADP-ribose of arginine), as opposed to HgCl2 or HCl (which act on ADP-ribosylated cysteine and serine/threonine residues, respectively), completely removed the ADP-ribose bound to the β subunit (Fig. 3B, lane 2), as we also observed with the [32P]ADP-ribosylated α subunit of G, induced by cholera toxin (Fig. 3B, lane 4). The possibility that the ADP-ribose is linked to glutamate could be ruled out since a 20-min treatment with NH2OH (a time sufficient to hydrolyze the ADP-ribose linked to glutamate) removed only 22% of the label from the ADP-ribo-
Few enzymes that catalyze ADP-ribosylation reactions have been purified and characterized, but among those the best known are the arginine-specific mono-ADP-ribosyltransferases. Most of the NAD:arginine ADP-ribosyltransferases purified so far are glycosylphosphatidylinositol (GPI)-anchored proteins located on the extracellular side of the plasma membrane or on the luminal face of intracellular organelles, thus being physically separated from intracellular substrates (43). The enzyme involved in subunit mono-ADP-ribosylation was markedly enriched in plasma membranes, from which it could not be separated by high salt, suggesting that it is an integral membrane protein. Moreover, phosphoinositide-specific phospholipase C, which hydrolyzes GPI-anchored membrane proteins (37), did not release the ADP-ribosyltransferase (whereas, as expected, it did release a GPI-anchored NADase in parallel experiments; data not shown). More importantly, intact cells were not able to induce ADP-ribosylation of the purified \( \beta \gamma \) subunit, whereas, under the same conditions, broken cells could (data not shown). These data indicate that the enzyme involved in the \( \beta \) subunit modification is not GPI-anchored and has a cytoplasm- or catalytic site.

This ADP-ribosyltransferase is present in most cell types, but the extent of \( \beta \) subunit labeling differed significantly among different cell lines (Fig. 1A). These differences might be due at least in part to the different rates of NAD\(^+\) metabolism in the cells examined, which in turn depend on the presence of membrane-associated NAD\(^+\)-utilizing enzymes such as ADP-ribosyltransferases, NADases, and NAD- and acid-pyrophosphatases. For instance, in FRTL-5 plasma membranes, which possess a NADase that rapidly and completely hydrolyzes NAD\(^+\) to ADP-ribose and nicotinamide (26), the ADP-ribosylation of the \( \beta \) subunit was barely detectable, whereas in CHO or HL60 plasma membranes, where 80% of the added NAD\(^+\) is still available after a 2-h incubation, the labeling of the \( \beta \) subunit was very pronounced (Fig. 1A).

It has been postulated that ADP-ribosylation is a reversible process in animal cells. ADP-ribosylarginine hydrolases are ubiquitous cytosolic enzymes that have been purified and cloned from rat brain (44) and subsequently from mouse and human brain (45). The rat hydrolase activity is Mg\(^2+\)- and thiol-dependent, whereas the recombinant human hydrolase activity is Mg\(^2+\)- but not thiol-dependent. In order to investigate whether the ADP-ribosylated \( \beta \gamma \) subunit could be a substrate of an arginine hydrolase, plasma membranes containing 12 ng of \(^{32}\)P-ADP-ribosylated \( \beta \) subunit were incubated with CHO cell cytosol in the presence of protease inhibitors (see "Experimental Procedures"). Fig. 4A shows that the cytosol was able to decrease the labeling of the \( \beta \) subunit (compare lanes 3 and 4) and that the concomitant addition of MgCl\(_2\) (lane 2) caused a further decrease; samples incubated with control buffer without or with MgCl\(_2\) (lanes 1 and 4, respectively) still contained 12 ng of modified \( \beta \) subunit, whereas the amount of modified \( \beta \) subunit was reduced to \(-9\) ng after the incubation with cytosol (lane 3) and to \(-6\) ng after the incubation with cytosol plus MgCl\(_2\) (lane 2) (as evaluated by InstantImager, Fig. 4D). Since the total amount of the \( \beta \) subunit was not affected during these de-ribosylation experiments (as demonstrated by Western blot analysis, Fig. 4B), we can conclude that a cytosolic protein, which could be Mg\(^2+\)-dependent, is able to decrease the ADP-ribosylation of the \( \beta \) subunit. The addition of DTT in the de-ribosylation experiments (not shown) did not affect the level of modified \( \beta \) subunit. HPLC analysis of the labeled compounds released from the \(^{32}\)P-ADP-ribosylated \( \beta \) subunit upon incubation with cell cytosol showed a peak of \(^{32}\)P-ADP-ribose (Fig. 4C, dashed line, \( E \)), indicating the activity of a cytosolic, ADP-ribosylarginine hydrolase. In the presence of MgCl\(_2\), which caused a further decrease in the labeling of the \( \beta \) subunit, there was not a parallel increase in \(^{32}\)P-ADP-ribose (Fig. 4C, continuous line, \( E \)). Under these conditions, the full HPLC analysis also revealed a large increase in \(^{32}\)P-AMP, thus indicating that the CHO cell cytosol still contains a Mg\(^2+\)-dependent pyrophosphatase, that releases \(^{32}\)P-AMP (derived both from the hydrolase-released \(^{32}\)P-ADP-ribose and directly from the \(^{32}\)P-ADP-ribosylated \( \beta \) subunit); a further phosphatase action on the

![Fig. 3. The G protein \( \beta \) subunit is ADP-ribosylated by an endogenous arginine-specific mono-ADP-ribosyltransferase.](http://www.jbc.org/Downloaded from by guest on July 24, 2018)
ribosylation of the ribosylation of GRP78/BiP protein (49). Thus the mono-ADP-[3H]adenine permits the study of toxin-catalyzed G proteins.

It has been previously shown that the labeling with cytosolically radiolabeled in order to study endogenous ADP-ribosylation in intact cells (30) and the metabolic conditions. Since NAD\(^+\) is a membrane-impermeant compound, it must either be injected into the cell or metabolically radiolabeled in order to study endogenous ADP-ribosylation. It has been previously shown that the labeling with \[^{32}P\]AMP would account for the \[^{32}P\]I formed. Indeed, it has been reported that pyrophosphatasases able to act on free ADP-ribose are present in the cytosol of different cells (46). Altogether these results are consistent with the proposal that the \(\beta\gamma\) subunit can undergo an endogenous ADP-ribosylation/deribosylation cycle. However, the possibility that the ADP-ribosylated \(\beta\) subunit might be processed not only by a hydrolase but also by a pyrophosphatase, as described in muscle cells for ADP-ribose. \[^{32}P\] radioactivity associated with labeled compounds was analyzed by scintillation counting. The inset shows a magnified HPLC elution pattern. D, level of ADP-ribosylated \(\beta\) subunit as measured by Instantimager. E, level of \[^{32}P\]ADP-ribose released as evaluated by HPLC analysis. The data shown in A–C are from a single experiment performed in duplicate, which is representative of at least three independent experiments. The data shown in D and E represent the mean of six independent experiments, performed with three different cytosol preparations.

\[^{32}P\]AMP; 2, AMP; 3, ADP-ribose. \[^{32}P\] radioactivity that is released in the supernatant of the samples shown in lane 2 (continuous line) and lane 3 (dashed line) of A and B during the deribosylation assay. Numbers indicate the elution positions of the different labeled compounds as identified by co-elution with commercially available standards. 1, AMP; 2, P; and 3, ADP-ribose. \[^{32}P\] radioactivity associated with labeled compounds was analyzed by scintillation counting. The inset shows a magnified HPLC elution pattern. D, level of ADP-ribosylated \(\beta\) subunit as measured by Instantimager. E, level of \[^{32}P\]ADP-ribose released as evaluated by HPLC analysis. The data shown in A–C are from a single experiment performed in duplicate, which is representative of at least three independent experiments. The data shown in D and E represent the mean of six independent experiments, performed with three different cytosol preparations.

\[^{32}P\]AMP; 2, AMP; 3, ADP-ribose. \[^{32}P\] radioactivity associated with labeled compounds was analyzed by scintillation counting. The inset shows a magnified HPLC elution pattern. D, level of ADP-ribosylated \(\beta\) subunit as measured by Instantimager. E, level of \[^{32}P\]ADP-ribose released as evaluated by HPLC analysis. The data shown in A–C are from a single experiment performed in duplicate, which is representative of at least three independent experiments. The data shown in D and E represent the mean of six independent experiments, performed with three different cytosol preparations.

The G Protein \(\beta\) Subunit Is ADP-ribosylated in Intact Cells—Due to the well recognized role that the G protein \(\beta\gamma\) subunit plays in cell regulation (48), it was important to analyze whether its mono-ADP-ribosylation could occur under physiological conditions. Since NAD\(^+\) is a membrane-impermeant compound, it must either be injected into the cell or metabolically radiolabeled in order to study endogenous ADP-ribosylation. It has been previously shown that the labeling with \[^{3}H\]adenine permits the study of toxin-catalyzed G proteins ADP-ribosylation in intact cells (30) and the \(\text{in vivo}\) ADP-ribosylation of GRP78/BiP protein (49). Thus the mono-ADP-ribosylation of the \(\beta\) subunit was analyzed by \(\text{in vivo}\) labeling of intact CHO cells employing \[^{3}H\]adenine to label metabolically the cellular NAD\(^+\) pool, followed by separation of the labeled \(\beta\) subunit with an affinity column (Fig. 5).

It is known that \[^{3}H\]adenine enters cells by carrier-mediated transport and is then phosphorylated to 5'-AMP which is in turn phosphorylated to ATP that enters the NAD\(^+\) pool (50). Fig. 5A shows that indeed \[^{3}H\]adenine is incorporated into intracellular NAD\(^+\) pool during the 16 h of labeling (see “Experimental Procedures”), being the NAD\(^+\)-associated cpm corresponding to 8% of the total cpm incorporated in the cells. The SDS-PAGE of \(^{3}H\)-labeled CHO cells extract (Fig. 5B, lane 1) revealed a major labeled band with a mass of approximately 70 kDa that may correspond to the modified GRP78/BiP protein (49), and some minor labeled bands. Among these, a 36-kDa band co-migrating with the ADP-ribosylated \(\beta\) subunit was observed. Moreover, as expected, treatment of \[^{3}H\]adenine-labeled CHO cells with pertussis toxin before extraction caused the labeling of the G protein \(\alpha\) subunits (Fig. 5B, lane 2). Thus, to explore the possibility that the labeled band of 36 kDa was the \(\beta\) subunit, crude membranes were prepared from \[^{3}H\]adenine-labeled CHO cell extract, and the \(\beta\) subunit they contain was affinity purified employing His\(_6\)-\(\alpha\) subunits (Fig. 5B, lane 1). As a control, 300 \(\mu\)g of \[^{3}H\]adenine-labeled CHO crude membranes were incubated with nickel-nitrilotriacetic acid-agarose resin (see “Experimental Procedures”). Fig. 5C shows the fluorography of \[^{3}H\]labeled \(\beta\) subunit obtained after its affinity purification (lane 1); as a control, 300 \(\mu\)g of \[^{3}H\]adenine-labeled CHO crude membranes were incubated with nickel-nitrilotriacetic acid-agarose resin only (lane 2), whereas lane 3 shows the labeling of 30 \(\mu\)g of the \[^{3}H\]adenine-labeled CHO crude membranes. From the densitometric analysis of the Western blot (Fig. 5D), we can conclude that \(\sim 2 \mu\)g of \(\beta\) were recovered from this affinity purification with \(\sim 0.2\%\) being modified in intact cells under basal condition. The possibility that the percentage of the modified \(\beta\) subunit will increase upon receptor activation is presently under investigation.

From these data it can be concluded that the substrate of endogenous ADP-ribosylation in CHO cells metabolically la-
beled with [3H]adenine is indeed the endogenous β subunit.

The β Subunit ADP-ribosylation Blocks the Inhibitory Effect of βγ on Type 1 Adenyl Cyclase—Free βγ subunit can directly activate several effectors (48), and it is generated upon ligand binding, when an activated heptahelix receptor causes GDP/GTP exchange on the G protein binding, when an activated heptahelix receptor causes GDP/GTP exchange on the G protein subunit, which then dissociates from both the receptor and the βγ dimer (48). This is not, however, the only process controlling the βγ state of activation. For instance, the RGS proteins (regulators of G protein signaling) increase the GTPase activity of the Go subunit, resulting in the accumulation of GDP-Go and its re-association with the free βγ subunit (51). Phosducin can bind to the βγ subunit directly and translocate it to the cytosol, thus preventing its association with Go or with effectors (52). In the present study we propose that mono-ADP-ribosylation might represent an additional mechanism to regulate directly the activity of the βγ subunit. The portions of the βγ subunit involved in establishing contact with the Go subunit and the effector proteins are increasingly being identified (53). Recent studies indicate that the amino-terminal region is involved not only in the interaction with the α subunit but also with adenylyl cyclase (54), phospholipase C-β2 (54), and the muscarinic atrial potassium channel (54). A small carboxyl-terminal segment of the β subunit has also been proposed to take part in the activation of phospholipase C-β2 (55). In order to determine whether the mono-ADP-ribosylation of the β subunit occurs on its amino- or carboxy-terminal moiety, we used a well established tryptic assay (29). The digestion of the βγ subunit by trypsin yields only two fragments with apparent molecular masses on SDS-PAGE of 14 (amino terminus) and 27 kDa (carboxyl terminus), which can also be identified by antibodies specific for the β amino and carboxyl termini. Trypsin cleavage of labeled membranes resulted in a reduced labeling of the 36-kDa protein and the appearance of a labeled ~14-kDa peptide (Fig. 6A, compare lane 2 with lane 5 and lane 3 with lane 6), which was recognized by the antibody specific for the amino-terminal fragment of the β subunit (Fig. 6B), indicating that this is the domain covalently modified by ADP-ribose. Following trypsin cleavage, purified bovine brain βγ (used as a control) was completely digested, yielding the two expected fragments of 27 and 14 kDa (compare lane 4 with lane 1 of Fig. 6, B and C), whereas the ADP-ribosylated endogenous β subunit (compare lane 6 with lane 3 of Fig. 6, B and C) or exogenously added purified βγ (compare lane 5 with lane 2 of Fig. 6, B and C) were partially digested, as clearly shown by immunoblot analysis, agreeing with previous observations that the membrane-associated βγ subunit is partially protected from trypsin degradation (56).

The amino-terminal portion of the β subunit encompassing amino acids 84–143 appears to be the region involved in the interaction with adenylyl cyclases (57). Interestingly, there are three arginine residues (96, 129, and 134) in the region predicted to interact with the 956–982 peptide of types 1 (AC1) and 2 (AC2) adenylyl cyclases (57). It is thus possible that arginine residues 96 and/or 129, which are located on the 14-kDa peptide produced by trypsin digestion of the βγ subunit, could be the target of the endogenous ADP-ribosyltransferase and that their modification might affect the interaction with adenylyl cyclase. Thus, in order to identify the site of modification on the β subunit, all arginine residues in the amino-terminal portion of this protein were mutated and replaced with lysine. The mutations were introduced into the background of rat βγ tagged at the amino terminus with the influenza hemagglutinin epitope. Addition of the hemagglutinin tag was important for the ADP-ribosylation assay in transfected CHO cells since the tagged β subunit, larger than the endogenous β subunit, could be easily identified on SDS-PAGE.

Indeed each β1 mutant was expressed in CHO cells with γ1 and tested for the ability to be endogenously ADP-ribosylated. All mutants could support some level of ADP-ribosylation, except the β1 mutant R129L, which was impaired in the ADP-ribosylation assay, thus indicating that indeed the endogenous ADP-ribosylation of the β subunit occurs on its arginine 129, one of the residues predicted to interact with adenylyl cyclases, as discussed above (57).

Therefore, we explored the possibility that this modification on arginine 129 of the β subunit could affect its ability to interact with AC1. Although the AC1 activity is absent in CHO membranes (the membrane associated to CHO plasma membranes), it can be efficiently stimulated by CaM and directly inhibited by βγ subunits (58). We thus used brain membranes as an AC1 source, and as expected, the unmodified βγ subunit partially inhibited CaM-stimulated AC1 in brain membranes (in agreement with previously reported data, Ref. 58), with this βγ-dependent inhibition being only slightly reduced after prolonged incubation of the β subunit at 37 °C (Fig. 6D). In contrast, under the same conditions the purified βγ subunit ADP-ribosylated in the presence of NAD+ and CHO membranes almost completely lost its inhibitory activity (Fig. 6D). In a series of control experiments, the βγ subunit incubated with CHO membranes without NAD+ (or with NAD− without membranes) was still able to inhibit AC1 activity (Fig. 6D); obviously, neither NAD+ and ADP-ribose nor CHO membranes alone had effects per se on the CaM-stimulated AC1 activity; finally, NAD+ added at the end of the incubation of the βγ
G Protein β Subunit ADP-ribosylation

subunit with CHO membranes (thus, under conditions that prevent ADP-ribosylation of the β subunit) did not interfere with βγ-dependent inhibition of AC activity. Altogether, these data indicate that the loss of activity of βγ is due to ADP-ribosylation of βγ by the membrane-associated ADP-ribosyltransferase. Furthermore, the loss of activity of the ADP-ribosylated β subunit was not due to a change in association to membranes, since equivalent amounts of the βγ subunit were found under all the experimental conditions employed (Fig. 6E, ADP-ribosylated βγ lane 1, unmodified βγ lane 2).

Notably, the loss of activity of the ADP-ribosylated βγ subunit on CaM-stimulated AC appears not to be complete; it was complete at 200 nM and decreased at higher βγ concentrations (Fig. 6D). This is probably due to the fact that not all of βγ is ADP-ribosylated; indeed, under our experimental conditions the amount of ADP-ribosylated βγ subunit (evaluated from the expected ~500-DA shift due to ADP-ribose on SDS-PAGE) was about 60–80% of the total βγ subunit (Fig. 6E). Thus, when 500 nM of “ADP-ribosylated” βγ was applied to the system, the unribosylated βγ amounted to 100–200 nM, a concentration sufficient to cause maximal inhibition of CAMP production. Moreover it can be demonstrated that the addition of ADP-ribosylated βγ (actual concentrations 10–200 nM) cannot affect the inhibition due to 200 nM of unmodified βγ, hence indicating that the ADP-ribosylated βγ is inactive on the inhibition of CAMP production (data not shown). This indicates that the ADP-ribosylated βγ does not per se affect the enzyme activity. However, whereas the precise mechanism remains to be defined, it is clear that ADP-ribosylation strongly affects the ability of βγ to interact with AC1.

As noted above, the βγ amino terminus is involved in interactions with the α subunit as well as with several effector proteins. It is therefore possible that amino-terminal ADP-ribosylation might be a general switch-off mechanism for βγ-mediated responses. Indeed, our preliminary data on the βγ-dependent activation of phospholipase C-β2 activity suggest that this is the case.2

Heterotrimeric G proteins are subjected to multiple modulatory inputs, as dictated by their central importance in cell regulation. Our finding of an ADP-ribosylation/deribosylation cycle acting on the β subunit delineates a novel molecular mechanism that could provide crucial control of G protein-mediated signaling pathways.

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Rosita Lupi, Daniela Corda and Maria Di Girolamo

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