ELSEVIER

FEBS Letters 338 (1994) 264–266

FEBS 13606

FEBS LETTERS

Pertussis toxin-catalyzed ADP-ribosylation of GTP-binding proteins with digoxigenin-conjugated NAD

Identification of the proteins in plasma membranes and nuclei

Yoshinori Takei[a], Katsunobu Takahashi[a], Yasunori Kanaho[a], Toshiaki Katada[a,b,*]

[a]*Department of Life Science, Tokyo Institute of Technology, Yokohama 227, Japan*
[b]*Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Tokyo 113, Japan*

Received 6 December 1993

Abstract

ADP-ribose moiety containing digoxigenin was transferred by pertussis toxin (IAP) to the α subunit of G_i ($G_i\alpha$) from digoxigenin-conjugated NAD (DIG-NAD) in a $\beta\gamma$ subunit-dependent manner ADP-ribosylation of $G_i\alpha$ with DIG-NAD *plus* IAP was inhibited by native NAD These results indicate that nonradiolabeled DIG-NAD also serves as the substrate for IAP-catalyzed ADP-ribosylation of G proteins Using DIG-NAD and fluorescein isothiocyanate-labeled anti-digoxigenin antibody, IAP-sensitive G protein(s) was found to be exist in nuclei as well as plasma membranes of rat liver and HeLa cells Thus, DIG-NAD is useful to identify pertussis toxin-substrate G proteins

Key words ADP-ribosylation, Digoxigenin, GTP-binding protein, Pertussis toxin

1. Introduction

ADP-ribosylation of $\alpha\beta\gamma$-trimeric GTP-binding proteins (G proteins) is catalyzed by several bacterial toxins [1]. It is well known that ADP-ribosylation of G proteins catalyzed by pertussis toxin (IAP) or cholera toxin modifies the function of G proteins· IAP-catalyzed ADP-ribosylation causes dissociation of G proteins (G_i and G_o) from receptors, thereby preventing the receptor-mediated signaling, while ADP-ribosylation of G_s by cholera toxin activates the protein [1] Thus, these toxins have been utilized to investigate the involvement of G proteins in signal transduction In addition to these toxins, endogenous mono-ADP-ribosyltransferases have recently been reported, suggesting that the endogenous ADP-ribosylation of G protein may regulate cellular signalings [2,3]

To date, ADP-ribosylation of G proteins has been studied by employing radiolabeled NAD such as [^{32}P]NAD, as a donor of ADP-ribose moiety If non-radiolabeled NAD analogs, which can serve as a substrate for toxin-catalyzed ADP-ribosylation of G proteins, are available, these compounds seem to be easy to handle, compared to [^{32}P]NAD with short half-life time It has recently been reported that biotinylated NAD substitutes for NAD in diphtheria toxin-catalyzed ADP-ribosylation of an elongation factor 2 [4] However, it is not clear whether biotinylated NAD also serves as a substrate for IAP- or cholera toxin-catalyzed ADP-ribosylation of G proteins Digoxigenin, a steroid isolated from digitalis plants, is used to conjugate with dUTP, and digoxigenin-conjugated dUTP can be a substrate of DNA polymerase [5] If IAP or cholera toxin transfers ADP-ribose moiety containing digoxigenin from digoxigenin-conjugated NAD (DIG-NAD) to G proteins, ADP-ribosylation of G proteins can be detectable with anti-digoxigenin antibody, which is now commercially available In the present study, we examined whether DIG-NAD serves as a substrate for IAP-catalyzed ADP-ribosylation of G_i Results here demonstrate that $G_i\alpha$ purified from bovine brain was specifically ADP-ribosylated with DIG-NAD *plus* IAP Furthermore, using this non-radiolabeled NAD analog, we indicate that IAP-substrate G protein(s) exists in nuclei as well as plasma membranes of rat liver and HeLa cells.

*Corresponding author Department of Life Science Tokyo Institute of Technology, Yokohama 227, Japan Fax (81) (45) 923 0367

Abbreviations G protein, GTP-binding protein, G_s, the stimulatory G protein of adenylyl cyclase, G_i, a family of homologous G proteins originally associated with inhibition of adenylyl cyclase, G_o, a G protein purified from bovine brain, DTT, dithiothreitol, IAP, islet-activating protein (pertussis toxin), SDS, sodium dodecyl sulfate, DIG-NAD, digoxigenin-conjugated NAD

SSDI 0014-5793(93)E1523-O

2. Experimental procedures

2 1 Preparation of DIG-NAD

To conjugate digoxigenin to NAD, 200 mM digoxigenin-3-*O*-methylcarbonyl-ε-aminocapronic acid-*N*-hydroxy-succinimide ester (Boehringer Mannheim) in dimethylfluoride and 20 mM N^6-[[(6-aminohexyl)carbamoyl]methyl]NAD (Sigma, A 5265) in 0 1 M Na-borate, pH 8 0, were mixed at molecular ratio of 2 1 and incubated overnight at 25°C in the dark The DIG-NAD was purified by column chromatography of DEAE-Toyopearl 650S (TOSOH) and AG-MP1 (Bio-Rad) The reaction mixture containing DIG-NAD was applied to DEAE-Toyoperl 650S and eluted with a linear gradient of 0–300 mM LiCl The fractions with DIG-NAD eluted about 100 mM LiCl were further applied to AG-MP1 column and eluted with 0–300 mM trifuluoroacetic acid The pure DIG-NAD eluted at about 75 mM trifuluoroacetic acid was lyophilized and stored at −80°C until use

2 2 Cell culture

HeLa cells (from Japanese Cancer Research Resources Bank) were grown in F-12 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C under an atmosphere of 95% air and 5% CO_2 The cells were passed and grown on glass coverslips (18 × 18 mm) 2 days before use for the following assay In Fig 2C, the HeLa cells were cultured with the medium containing 250 ng of IAP/ml for the last 6 h of the culture

2 3 IAP-catalyzed ADP-ribosylation of G_i

The purification of G_i from bovine brain and the separation of α and βγ subunits were described previously [6] The purification of rat liver membranes and nuclear envelopes was described previously [7] IAP was preactivated by incubating at 37°C for 10 min in 50 mM Tris-HCl, pH 7 5, containing 100 mM DTT and 0 1 mM ATP Samples were incubated at 30°C for 30–40 min with the preactivated IAP (20 ng/ml) in 25 μl of reaction mixture consisting of 100 mM Tris-HCl, pH 7 5, 5–10 μM DIG-NAD, 1 mM EDTA, 40 μM GDP, 0 1 mM NADP, 10 mM thymidine, 1 mM ADP-ribose, and 20 mM nicotinamide [7] In some experiments, the samples were further treated with 20 U/ml of phosphodiesterase (Sigma, P 6877) at 37°C for 1 h The reaction was terminated by adding equal volume of 2-fold concentrated Laemmli-buffer, followed by boiling at 90°C for 3 min Samples were subjected to SDS-polyacrylamide gel (12%) electrophoresis and then blotted to polyvinylidene difluoride membranes (Bio-Rad) The blotted membranes were washed with buffer A (50 mM Na-HEPES, pH 7 4, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 2 mM DTT and 0 5 mM EGTA) and incubated in a blocking solution (buffer A containing 10% heat inactivated fetal calf serum, 0 1% bovine γ-globulin and 0 2% NP-40) at room temperature After 1 h, the horse radish peroxidase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) was added and incubated for 30 min at room temperature The digoxigenin-labeled proteins were detected with ECL Western blotting detection system (Amersham) or POD-immunostain set (Wako)

2 4 Cellular distribution of IAP-substrate G proteins in HeLa cells

To study the cellular distribution of IAP-substrate G proteins, HeLa cells grown on coverslips were permeabilized with buffer A containing 100 kallikrein inhibitory units of aprotinin/ml, 10 μg of leupeptine/ml and 150 μg of digitonin/ml for 10 min on ice After removing the digitonin solution, the cells were treated with IAP as described above After 40 min, the cells were washed with buffer A, fixed with 3 7% formaldehyde, then incubated for 30 min at room temperature in the blocking solution, followed by the treatment of fluorescein isothiocyanate-labeled anti-digoxigenin antibody (Boehringer Mannheim) for 15 min The cells were mounted on a slide glass with a small amount of buffer A containing 1 mg of phenylenediamine/ml and 10% glycerol, and the fluorescent images were observed under the Nikon Microphot-FX microscope

2 5 Miscellaneous

The concentration of proteins was determined by the method of Lowry et al [8] with bovine serum albumin as a standard

3. Results and discussion

3 1 DIG-NAD serves as a substrate for IAP-catalyzed ADP-ribosylation

When G_i purified from bovine brain was incubated with DIG-NAD *plus* IAP and then analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting, only the α subunit of G_i ($G_i\alpha$) was detected with anti-digoxigenin antibody (Fig 1A) The extent of labeling was significantly reduced by further incubation with phosphodiesterase, demonstrating that ADP-ribose moiety containing digoxigenin was transferred to $G_i\alpha$ by IAP The mode of ADP-ribosylation with DIG-NAD was similar to that with [^{32}P]NAD, since ADP-ribosylation of $G_i\alpha$ with DIG-NAD, as well as that with [^{32}P]NAD, absolutely required βγ subunits (Fig 1B) and was inhibited with increasing concentrations of NAD (Fig 1C). The half-maximum inhibition by native NAD of ADP-ribosylation with DIG-NAD was observed at the almost same concentration as that of DIG-NAD Thus, DIG-NAD, as well as [^{32}P]NAD, serves as a substrate for IAP-catalyzed ADP-ribosylation of $G_i\alpha$.

3 2 Cellular distribution of IAP-sensitive G proteins

IAP-catalyzed ADP-ribosylation with DIG-NAD, as

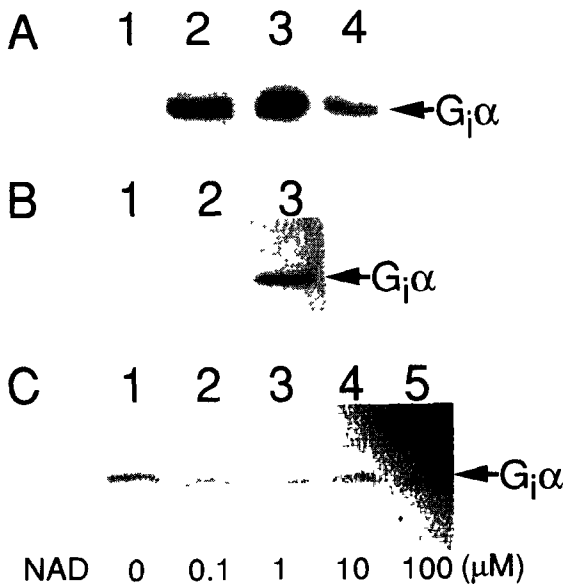

Fig 1 ADP-riboslation of the α subunit of G_i with DIG-NAD (A) G_i (2 pmol) purified from bovine brain was incubated with 10 μM DIG-NAD in the absence (lane 1) or presence of IAP (lane 2) at 30°C for 40 min G_i (4 pmol) incubated with IAP plus DIG-NAD was further treated without (lane 3) or with (lane 4) phosphodiesterase as described in section 2 3 (B) α Subunit of G_i ($G_i\alpha$, 1 pmol, lane 1), βγ subunits (1 pmol, lane 2) or $G_i\alpha$ plus βγ subunits (lane 3), which were purified from bovine brain, was incubated with IAP plus 5 μM DIG-NAD as described in section 2 3 (C) G_i purified from bovine brain was incubated with IAP plus 10 μM DIG-NAD in the presence of the indicated concentrations of native NAD for 40 min at 30°C Samples were subjected to SDS-polyacrylamide gel (12%) electrophoresis and then blotted to the polyvinylidene difluoride membranes Proteins ADP-ribosylated with DIG-NAD were detected by the POD-immunostain set

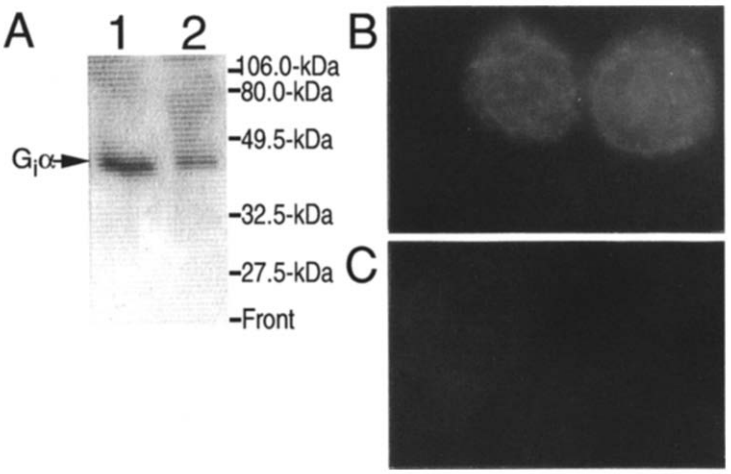

Fig 2 Cellular distribution of IAP-substrate G proteins (A) Membranes (20 μg protein, lane 1) or nuclear envelopes (40 μg protein, lane 2) prepared from rat liver were incubated with IAP plus 5 μM DIG-NAD as described in section 2 3 Western-blotting system with ECL was used to detect the ADP-ribosylated proteins (B and C) HeLa cells which had been cultured without (panel B) or with (panel C) IAP were permeabilized with digitonin and then treated with DIG-NAD plus IAP as described in section 2 4 Bar = 10 μm

well as ADP-ribosylation with [^{32}P]NAD [7], was highly specific to a 40-kDa IAP-substrate G protein, since only a 40-kDa protein was ADP-ribosylated when rat liver membranes were incubated with DIG-NAD *plus* IAP (Fig 2A, lane 1) Under the same conditions, it was found that in the nuclear envelope fraction of rat liver a 40-kDa protein was ADP-ribosylated with DIG-NAD *plus* IAP, consistent with the idea that nuclei also contains an IAP-substrate G protein, as suggested previously by us [7] (Fig 2A, lane 2) Highly specific ADP-ribosylation of the 40-kDa protein with DIG-NAD led us to study the cellular distribution of IAP-substrate G proteins by immunofluorescent assay As shown in Fig 2B, the fluorescent signals were observed around plasma membranes as dispersed dots and around nuclear envelopes as a rim These fluorescent signals were almost completely abolished when the cells which had been cultured with IAP were subjected to the immunofluorescent assay (Fig 2C), indicating that fluorescent signals represent the location of the IAP-substrate The fluorescent rim around the nucleus seems to confirm the localization of IAP-substrate in the nuclei This image is very similar to that of the binding of fluorescent nuclear localization signal-containing proteins to the nuclei [9], supporting the idea describing above In contrast, the fluorescent signals around plasma membranes were dispersed This result suggests that G_i is located as clusters in plasma membranes Alternatively, dispersed dot signals of plasma membranes may be responsible for artifact permeabilization by digitonin before ADP-ribosylation may simultaneously solubilize some amount of plasma membrane G_i and subsequent removal of digitonin solution also washes out the solubilized G_i In contrast to plasma membrane G proteins, IAP-substrate G protein in nuclei seems to be resistant to detergents, since 1% Triton X-100 failed to extract nuclear G protein [7]. Recently, it has been reported that IAP-sensitive G protein, G_i, locates at the endosome and Golgi body [10], as well as the plasma membranes and nuclei In our conditions, however, these organelles could not be detected by anti-digoxigenin antibody

In summary, we represented that non-radiolabeled DIG-NAD, serves as a substrate for IAP-catalyzed ADP-ribosylation Using DIG-NAD, the existence of IAP-substrate G protein in nuclei was demonstrated, consisting with the idea of our previous paper [7]

Acknowledgements We would like to thank Dr Hiroshi Nishina for helpful discussion

References

[1] Gilman, A G (1987) Annu Rev Biochem 56, 615–649
[2] Maehama, T , Takahashi, K , Ohoka, Y , Ohtsuka, T , Ui, M and Katada, T (1991) J Biol Chem 266, 10062–10065
[3] Zolkiewska, A , Nightingale, M S and Moss, J (1992) Proc Natl Acad Sci USA 89, 11352–11356
[4] Zhang, J and Snyder, S H (1993) Biochemistry 32, 2228–2233
[5] Escarceller, M , Rodriguez-Frias, F , Jardi, R , Segundo, B S and Eritja, R (1992) Anal Biochem 206, 36–42
[6] Kobayashi, I , Shibasaki, H , Takahashi, K , Tohyama, K , Kurachi Y , Ito, H , Ui, M and Katada, T (1990) Eur J Biochem 191 499–506
[7] Takei, Y , Kurosu, H , Takahashi, K and Katada, T (1992) J Biol Chem 267, 5058–5089
[8] Lowry, O H , Rosebrough, N J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265–275
[9] Moore, M S and Blobel, G (1992) Cell 69, 939–950
[10] Carter, L L , Redelmeier, T E , Woollenweber, L A and Schmid, S L (1993) J Cell Biol 120, 37–45