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Analysis of guanine nucleotide bound to *ras* protein in PC12 cells

Takaya Satoh, Masami Endo, Shun Nakamura and Yoshito Kaziro

Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minatoku, Tokyo 108, Japan

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The *ras* gene product (p21) specifically binds GDP or GTP. In analogy with the reaction mechanism of other GTP-binding proteins, only the GTP-bound conformation is believed to be the biologically active one. Previously, we reported that not only oncogenic p21(Val-12) but also proto-oncogenic p21(Gly-12) could induce morphological differentiation in rat pheochromocytoma PC12 cells when microinjected in the complexed form with GTPγS [(1987) Mol. Cell. Biol. 7, 4553–4556]. In the present report we transformed PC12 cells with the oncogenic *ras* gene placed under the metallothionein I promoter. It was found that the transformed cells, when induced with Cd^{2+}, differentiated in the absence of NGF. Then we analyzed the guanine nucleotide bound to p21 in the intact PC12 cells. It was found that conditionally induced p21(Val-12) was mostly present in the GTP-bound form, whereas the endogenous p21(Gly-12) was in the GDP-bound form. These results indicate again that p21·GTP induces the morphological differentiation of PC12 cells.

ras protein; Nucleotide exchange; (PC12 cell)

1. INTRODUCTION

A *ras* gene family consisting of Harvey (Ha)-, Kirsten (Ki)- and N-*ras* has been detected as active transforming genes, believed to play an essential role in growth or differentiation of a variety of cells (review [1]). The *ras* protein (p21) specifically binds guanine nucleotides and hydrolyzes bound GTP to GDP and P_i. The GTPase activity of transforming protein mutated at position 12 from Gly to Val is severely impaired [1]. From these biochemical properties it is proposed that p21 may function in an analogous manner to other GTP-binding proteins, such as translational factors [2] and G-proteins [3]. The binary p21·GTP complex is an active conformation in promoting signal transduction whereas the complex consisting of p21·GDP is inactive.

In a previous report [4], we studied the effect of GDP and GTP bound to p21 on inducing neurite formation in rat pheochromocytoma PC12 cells using the microinjection technique. Normal p21(Gly-12) was unable to induce morphological changes when injected in the form of a p21·GDP complex, but p21(Gly-12) preincubated with GTPγS or GTP efficiently induced differentiation. p21(Val-12)·GDP also efficiently induced neurite extension. We suppose that in this case p21(Val-12)·GTP may be formed by nucleotide exchange in the cell, and because of its impaired GTPase activity, may exist stably to transduce the differentiation signal. Our observation that excess amounts of GDPβS coinjected with p21(Val-12)·GDP inhibited the neurite extension by the latter supports this idea.

Here, we analyzed p21-bound guanine nucleotides in PC12 cells using an improved procedure. It was found that conditionally expressed exogenous p21(Val-12) bound mainly GTP and was able to induce the morphological conversion, whereas the endogenous normal p21(Gly-12) mostly bound GDP. This observation provides strong support for p21(Val-12) causing the morphological conversion of PC12 cells since it exists in a GTP-bound conformation in the cells.

Correspondence address: T. Satoh, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minatoku, Tokyo 108, Japan

Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate)

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2. MATERIALS AND METHODS

2.1. *Cell culture and induction of p21(Val-12)*

PC12 and PC12-MTHR(Val-12) (see below) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (MA Bioproducts) and 5% horse serum (Gibco) under 5% CO_2 at 37°C. The *ras*(Val-12) gene in PC12-MTHR(Val-12) cells was induced by adding 1 μM $CdCl_2$ to the culture medium.

2.2. *Plasmid construction and transfection*

The detailed procedure for construction of the expression plasmid pMT-cHr(V)-neo will be reported elsewhere (Endo et al., unpublished). pMT-cHr(V)-neo contains the *neo*^{r} gene from pSV2-neo [5] and the human c-Ha-*ras* cDNA mutated at position 12 (from Gly to Val) which was placed under the control of mouse metallothionein I promoter (1.9 kb *Eco*RI-*Bgl*II fragment from the 5′-untranslated region [6]). The splice signals and the poly(A) addition site of SV40 were ligated downstream of the human c-Ha-*ras* cDNA.

pMT-cHr(V)-neo was cut with a unique *Eco*RI site and was introduced into PC12 cells by a conventional DNA transfection method [7]. G418-resistant colonies were selected [5] and 12 clones were screened for the morphological changes induced by $CdCl_2$. One clone designated as PC12-MTHR(Val-12) was used for further analysis.

2.3. *Immunoprecipitation and Western blotting*

A membrane-rich fraction was prepared as described [8] and dissolved in RIPA buffer [50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.8% NP-40, 0.8% deoxycholate, 0.08% SDS, 0.8 mM phenylmethylsulfonyl fluoride]. Anti-p21 monoclonal antibody Y13-238 [9], rabbit anti-rat IgG antiserum (Cappel) and protein A-Sepharose CL-4B (Sigma) were suspended in RIPA buffer, mixed mildly at 4°C for 2 h and the precipitate washed three times with RIPA buffer. The membrane-rich lysate was added to the antibody-protein A-Sepharose complex and mixed at 4°C for 2 h. The precipitate was then washed four times with RIPA buffer and dissolved in Laemmli sample buffer [10]. SDS-polyacrylamide gel electrophoresis and Western blotting were carried out as in [11].

2.4. *Analysis of GDP and GTP bound to p21*

Cells were seeded at a density of 10^4 cells/cm^2 on 100-mm dishes and cultured for 4 days in the presence or absence of 1 μM $CdCl_2$. The cells were labeled for 2 h with 0.6 mCi/ml [^{32}P]orthophosphate (NEN, NEX-053, 100 mCi/ml) in phosphate-free DMEM supplemented with 10% fetal calf serum and 5% horse serum, which had been dialyzed against 0.9% NaCl to eliminate phosphate ions. The cells were washed twice with the phosphate-free medium and then twice with Tris-buffered saline [50 mM Tris-HCl (pH 7.5), 150 mM NaCl], scraped off with a rubber policeman and harvested by centrifugation in a 1.5 ml plastic tube. Thereafter, all procedures were carried out at 4°C. To the cell pellet 0.6 ml of immunoprecipitation buffer was added, containing 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 150 mM NH_4Cl, 0.5% NP-40, and 1% aprotinin. The suspension was gently inverted for 10 min and centrifuged at 2500 rpm for 5 min. To the supernatant was added charcoal precoated with bovine serum albumin and suspended in immunoprecipitation buffer. After vigorous mixing, the charcoal was removed by centrifugation and the lysate stored at −80°C until use.

Anti-p21 monoclonal antibody Y13-259 or Y13-238 [9] was mixed with rabbit anti-rat IgG antiserum (Cappel) and protein A-Sepharose CL-4B (Sigma), the mixture being incubated at 0°C for 15 min. In control experiments, 10 μg recombinant p21(Gly-12) [11] was also included to absorb the anti-p21 antibody in advance. The cell lysate was added to the antibody-protein A-Sepharose complex and mixed gently for 1 h. After centrifugation the precipitate was washed twice with immunoprecipitation buffer and then five times with washing buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$ and 150 mM NH_4Cl. The precipitate was suspended in 20 μl of a mixture containing 20 mM Tris-HCl (pH 7.5), 20 mM EDTA, 2% SDS, 0.5 mM GDP and 0.5 mM GTP and heated at 65°C for 5 min. The supernatant was spotted onto a polyethyleneimine-cellulose thin-layer plate and developed with 0.5 M KH_2PO_4 (pH 3.4). The positions of GDP and GTP were assigned by visualization of unlabeled marker under ultraviolet light and the plate was subjected to autoradiography.

3. RESULTS AND DISCUSSION

To study the differentiation induced by *ras* protein, we cloned a PC12 cell line that conditionally expresses Ha-*ras*(Val-12). The structure of the expression vector is shown in fig.1. Human c-Ha-*ras*(Val-12) cDNA was placed under the mouse metallothionein I promoter, which can be activated by heavy-metal ions [12]. The bacterial *neo*^{r} gene was also present on the same plasmid as a selection marker. The plasmid was introduced into PC12 cells and the transformants selected using G418. From G418-resistant transformants, we selected clones which normally grow in the absence of $CdCl_2$, but change morphology to neurite-

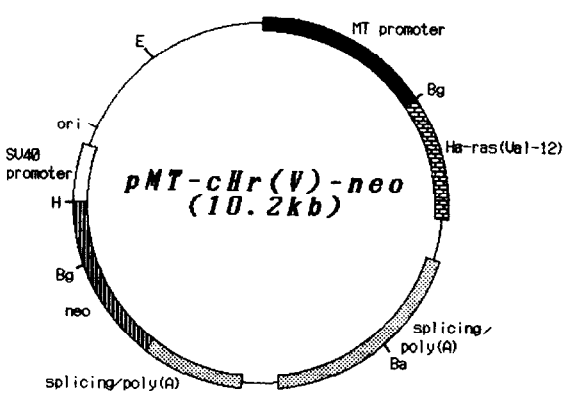

Fig.1. Structure of the expression plasmid pMT-cHr(V)-neo. Restriction enzymes: Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII. MT promoter, mouse metallothionein I promoter; *ori*, replication origin of pBR322.

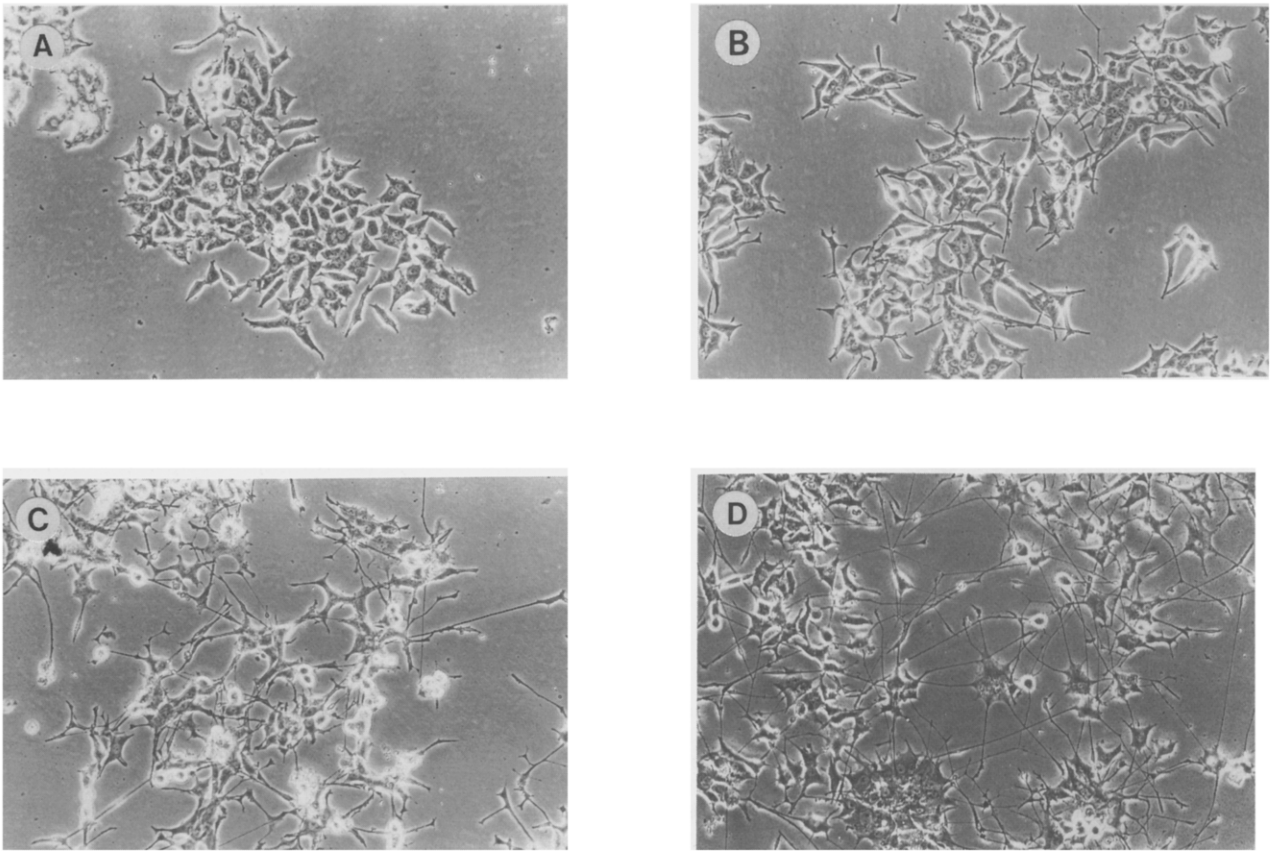

Fig.2. Morphological change of PC12-MTHR(Val-12) cells. (A) PC12-MTHR(Val-12) cells growing in the absence of $CdCl_2$. (B,C) PC12-MTHR(Val-12) cells cultured in the presence of 1 μM $CdCl_2$ for 2 days (B) and 8 days (C), respectively. (D) PC12 cells cultured in the presence of 50 ng/ml nerve growth factor (2.5 S NGF, Takara Shuzo, Japan) for 6 days.

bearing cells on addition of 1 μM $CdCl_2$. One such cell line, termed PC12-MTHR(Val-12), was studied further. The presence of 1 μM $CdCl_2$ had no effect on growth or morphology of parental PC12 cells (not shown).

The morphological change in PC12-MTHR(Val-12) induced by $CdCl_2$ is shown in fig.2. Processes appeared approx. 36 h after the addition of $CdCl_2$, later than in the case of nerve growth factor (NGF). Since the change could be observed after 12 h when recombinant p21 was injected into PC12 cells (not shown), this lag period may be required for expression of the Ha-*ras* gene and accumulation of the protein products. After 2 days, we were able to identify clearly the change in morphology (fig.2B), and after 8 days, long branched neurites, similar to those of NGF-treated PC12 cells, were observed in more than 90% of the cells (fig.2C,D).

The amounts of p21 in induced and uninduced cells were estimated as follows. p21 was first immunoprecipitated from the lysate with anti-p21 monoclonal antibody Y13-238 and then detected by Western blotting. As shown in fig.3, a 21 kDa band was stained with rabbit anti-p21 polyclonal antibody Has 6 [13], but not with normal rabbit serum (not shown). Increased amounts of p21 were found in PC12-MTHR(Val-12) cells compared with that of endogenous p21 in PC12 cells when cultured for 4 days in the presence of $CdCl_2$. The amounts of p21 in PC12-MTHR(Val-12) cells were higher than in parental PC12 cells even in the absence of $CdCl_2$. This may be due to the leaky expression from the metallothionein promoter

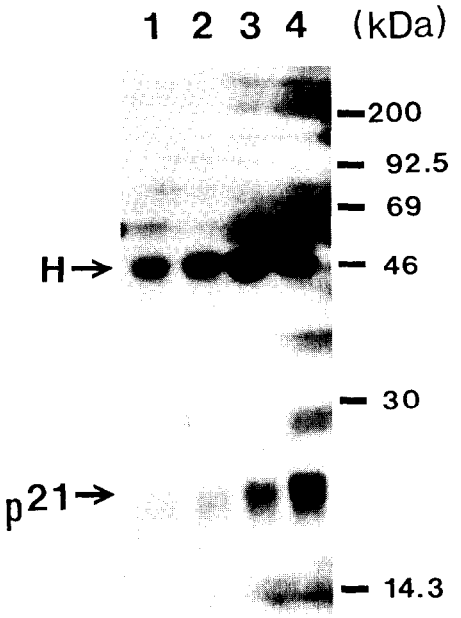

Fig.3. Induction of p21 in PC12-MTHR(Val-12) cells. Cells were cultured in the presence (lanes 2,4) or absence (lanes 1,3) of 1 μM $CdCl_2$ for 4 days. A lysate of the membrane-rich fraction was prepared and p21 was immunoprecipitated with Y13-238 from PC12 (lanes 1,2) or PC12-MTHR(Val-12) (lanes 3,4) cells as described in section 2. After SDS-polyacrylamide gel electrophoresis (200 μg membrane-rich fraction per lane), p21 was immunologically detected with Has 6 [13] and ^{125}I-protein A (Amersham IM.144, 3 μCi/ml). H, immunoglobulin heavy chain.

without inducers [6]. Swiss 3T3 cells transfected with the same plasmid pMT-cHr(V)-neo also expressed Ha-*ras* mRNA in a larger quantity than parental cells without $CdCl_2$ (Endo et al., unpublished).

Analysis of GDP and GTP bound to p21 was carried out using parental PC12 and PC12-MTHR(Val-12) cells. Cells were cultured for 4 days in the presence of 1 μM $CdCl_2$ and labeled with [^{32}P]orthophosphate for 2 h. The cells were then dissolved in a buffer containing 20 mM $MgCl_2$ and 0.5% NP-40, and p21 was immunoprecipitated with anti-p21 monoclonal antibodies. As shown in fig.4, we could detect labeled GDP and GTP in the immunoprecipitates formed with Y13-259 or Y13-238, but not with normal rat IgG. Since these spots disappeared completely when the antibody had been neutralized with excess amounts of recombinant p21 (fig.4A,C), it is certain that the nucleotides were derived from p21.

In parental PC12 cells, most of the endogenous p21 bound GDP. $CdCl_2$ had no effect on the GDP/GTP ratio with the parental PC12 cells (not shown). In contrast, a significant amount of GTP was observed when p21(Val-12) was induced by $CdCl_2$ in PC12-MTHR(Val-12) cells. More GTP-bound p21 was immunoprecipitated by Y13-238 than by Y13-259 (fig.4A,C). The reason for this quantitative difference is not clear.

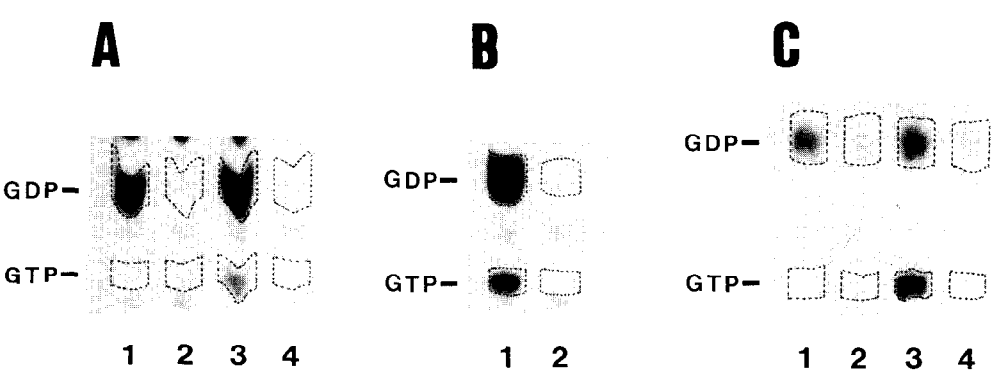

Fig.4. Analysis of GDP and GTP bound to p21. Cells were cultured in the presence of 1 μM $CdCl_2$ for 4 days. p21 was immunoprecipitated from ^{32}P-labeled cell lysate and the nucleotides bound to p21 were analyzed by thin-layer chromatography as described in section 2. In panels A and C, the lysate was prepared from PC12 (lanes 1,2) or PC12-MTHR(Val-12) (lanes 3,4) cells, divided into two aliquots and immunoprecipitation was performed with (lanes 2,4) or without (lanes 1,3) 10 μg recombinant p21(Gly-12) [11]. Antibodies used were Y13-259 (A) and Y13-238 (C). In (B), the lysate of PC12-MTHR(Val-12) cells was divided into two aliquots and p21 was immunoprecipitated with Y13-259 (lane 1) or rat normal IgG (Alfacell) (lane 2). Trichloroacetic acid-insoluble counts of the cell lysate per lane were the same in each panel; (A) 2.0×10^7 cpm/lane; (B) 2.9×10^7 cpm/lane; (C) 1.4×10^7 cpm/lane. Dotted squares indicate the positions of GDP or GTP as internal markers.

High concentrations of $MgCl_2$ inhibit the dissociation of bound guanine nucleotides from p21 [11,14]. In a separate experiment (not shown), we confirmed that all of the radioactivity of [^{3}H]GDP bound to recombinant p21 can be recovered by immunoprecipitation under the same conditions as in fig.4, indicating that the p21·GDP complex did not dissociate during the immunoprecipitation. Furthermore, the fact that excess amounts of recombinant p21 could extinguish labeled GDP and GTP, though ^{32}P-labeled free GDP and GTP were present in the cell lysate, indicates that nucleotide exchange did not occur during the procedure. Therefore, we consider that the results shown in fig.4 represent the association of GDP or GTP to p21 in vivo. It should be noted that the charcoal treatment of the cell lysate prior to immunoprecipitation is remarkably effective in reducing the background radioactivity in thin-layer chromatography.

From the above results, we can conclude that at least a certain population of p21(Val-12) binds GTP in PC12 cells, whereas endogenous p21 binds only GDP. In [4], we observed that p21(Gly-12)·GTP and p21(Gly-12)·GTPγS but not p21(Gly-12)·GDP could induce morphological differentiation in PC12 cells when injected into the cells. On the other hand, p21(Val-12)·GDP was able to induce morphological conversion, and an excess amount of GDPβS coinjected with p21(Val-12)·GDP inhibited the differentiation. We suppose that the injected p21(Val-12) may be converted to the GTP-bound form and remains in the active conformation. Our present results that p21(Val-12) actually binds GTP in the cells strongly support this contention.

Trahey and McCormick [15] reported similar results in maturation of *Xenopus* oocyte. They showed that the injected p21 could induce maturation of the oocytes only in the GTP-bound conformation, and that the injected transforming p21 mainly bound GTP in contrast to normal p21 that bound GDP in the cells. Gibbs et al. [14] also analyzed guanine nucleotides bound to yeast and mammalian *ras* proteins expressed in yeast cells and found that the amount of GTP-bound form was correlated to the activity to bypass the *cdc25* mutation. Our results suggest that, also in the case of mammalian cells, the mechanism of activation of p21 by substitution at position 12 (from Gly to Val) is that the mutated p21 is present stably in the GTP-bound conformation. The observation of Trahey and McCormick [15] that the GTPase activating protein (GAP), which specifically activates the GTPase of the normal p21, could also be detected in mammalian cells is well consistent with our results.

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