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Differential expression of α-subunits of G-proteins in human neuroblastoma-derived cell clones

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The distribution of α- and β-subunits of G-proteins was analyzed in membranes of three cell clones which are derived from the human neuroblastoma cell line SK-N-SH. The neuroblast-like clone SH-SY5Y shows a pattern of G-proteins very similar to that of human brain cortex with high levels of $G_{i\alpha}$ and $G_{o\alpha}$ but low levels of $G_{40\alpha}$. The intermediate clone SH-IN contains high levels of $G_{o\alpha}$ and $G_{i\alpha}$ and moderate levels of $G_{40\alpha}$. The non-neuronal clone SH-EP shows high levels of $G_{40\alpha}$ but lacks $G_{o\alpha}$. Differentiation of the neuroblast-like clone SH-SY5Y by retinoic acid or nerve growth factor does not change the amount of $G_{i\alpha}$ or $G_{o\alpha}$ in the membrane.

G-protein; Immunoblot analysis; Differentiation; Retinoic acid; Nerve growth factor; (Human neuroblastoma cell)

1. INTRODUCTION

A family of GTP-binding proteins (G-proteins) transduces signals from membrane-bound receptors to intracellular effector systems (review [1]). Functionally characterized members of this family include the stimulatory (G_s) and inhibitory (G_i) GTP-binding proteins that couple hormone and neurotransmitter receptors to the adenylate cyclase system. These proteins are heterotrimers composed of α-, β- and γ-subunits. The α-subunits vary in size from 39 to 52 kDa and can be ADP-ribosylated by pertussis toxin and/or cholera toxin. They also contain the GTP-binding site and are endowed with a GTPase activity. Recently porcine brain membranes were shown to contain three different GTP-binding proteins ($G_{i\alpha}$, $G_{40\alpha}$, $G_{o\alpha}$) that can be specifically ADP-ribosylated by pertussis toxin [2]. There is increasing evidence that G-proteins that are substrates of pertussis toxin, are also involved in coupling of receptors to ion channels [3] and phospholipase C [4].

Human neuroblastoma cells show diverse morphological and biochemical properties, with characteristics of cells derived from the neural crest which develop into neurons, Schwann cells and melanocytes [5]. The human neuroblastoma cell line SK-N-SH contains at least two morphologically distinct types of cells [6]. One cell type is neuroblast-like with a small cell body that may extend short neuritic processes and is weakly substrate adherent. The other cell type is a flat non-neuronal cell with strong substrate adhesiveness. Biedler and co-workers [7,8] have selected cloned neuronal and non-neuronal cell lines from SK-N-SH cells that are relatively stable phenotypically although they can interconvert at a very slow

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Abbreviations: G-protein, guanine nucleotide-binding protein; DTT, dithiothreitol; NAD, nicotinamide adenine dinucleotide; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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rate. Subclones include SH-SY5Y (neuroblast-like), SH-IN (intermediate) and SH-EP (non-neuronal). In addition to morphological differences these subclones also exhibit dramatic differences in the expression of neurotransmitter systems. For example, the μ- and δ-opioid receptor, negatively coupled to adenylate cyclase [9], and the muscarinic receptor stimulating phosphatidylinositol turnover [10], are strongly expressed in the neuroblast-like clones SH-SY5Y and SH-IN, but absent or barely detectable in the non-neuronal clone SH-EP [11]. Because of the differences in neurotransmitter receptor expression we have investigated the composition of G-proteins in subclones derived from the human neuroblastoma cell line SK-N-SH utilizing both pertussis toxin catalyzed ADP-ribosylation and immunoblot analysis with several specific antibodies.

2. MATERIALS AND METHODS

2.1. *Culture and treatment of human neuroblastoma-derived cell clones*

The SK-N-SH cell line and its subclones (SH-SY5Y, SH-IN, SH-EP) were provided by Dr June L. Biedler of the Sloan-Kettering Institute for Cancer Research, Rye, NY. The cells were grown at 37°C as monolayer in RPMI 1640 medium supplemented with 10% fetal calf serum in the presence of 100 μg streptomycin/ml and 100 IU penicillin/ml. The differentiation of SH-SY5Y cells was carried out by culturing the cells (in 80% confluency) with either 50 ng/ml nerve growth factor (2.5 S form, Sigma, St Louis, MO, USA) or 10 μM retinoic acid (Sigma, USA) for 6 days. Medium was replaced every 2 days. In the case of retinoic acid-treated cells, equal ethanol concentrations were present in the control cells. Confluent cells were harvested with 0.04% EDTA in Ca^{2+}/Mg^{2+} free phosphate-buffered saline with mechanical agitation. The cell suspension was centrifuged at 1000 rpm for 7 min and the pellets were collected and stored at −75°C.

2.2. *Membrane isolation and ADP-ribosylation*

Membranes were prepared by Dounce homogenization of cells and human brain frontal lobe cortex as described in [12]. Proteins were assayed by the method of Peterson [13] using bovine serum albumin as a standard. 25 μg membrane proteins were ADP-ribosylated in a total volume of 50 μl containing 100 mM Tris-HCl, pH 7.8, 1 mM EDTA, 2.5 mM $MgCl_2$, 10 mM DTT, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 0.5 μg pertussis toxin (List Biological Laboratories, Campbell, CA, USA) and 2 μCi [α-^{32}P]NAD (800–1000 Ci/mmol, NEN, Dreieich, FRG). The reaction was allowed to proceed for 60 min at 32°C, stopped by the addition of an equal volume of two-fold concentrated sample buffer (0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue) and heated for 5 min at 95°C.

2.3. *SDS-polyacrylamide gel electrophoresis and autoradiography*

ADP-ribosylated membrane proteins were analyzed on SDS-polyacrylamide gradient gels prepared by the method of Laemmli [14]. The slab gels (1.5 mm thick) consisted of a 10–12% separating gel and a 5% stacking gel. Molecular mass standards were [^{14}C]methylated proteins (Amersham Buchler, Braunschweig, FRG). After electrophoresis the gels were dried and exposed to Hyperfilm-βmax (Amersham Buchler).

2.4. *Preparation of antibodies against subunits of G-proteins*

Antibodies against the GTP-binding site of α-subunits from G-proteins ($G_{\alpha common}$), against $G_{o\alpha}$ and G_{β} were prepared as described by Mumby et al. [15]. A new antiserum specifically directed against the α-subunit of the 40 kDa G-protein was prepared by immunizing rabbits with a synthetic peptide derived from the amino acid sequence [3–17] of rat cDNA clone λGX13, which has been reported to code for $G_{i\alpha}$ [16]. Immunoblot analysis of purified G-proteins from bovine brain indicates that this antiserum does not recognize $G_{i\alpha}$ but reacts with $G_{40\alpha}$. The preparation and characterization of the antiserum are described elsewhere (Lang and Costa, submitted).

2.5. *Immunoblot analysis of SDS-polyacrylamide gels*

Membrane proteins and prestained protein molecular mass standards (BRL, Eggenstein, FRG) were separated on SDS-polyacrylamide gradient gels and then electrotransferred onto

nitrocellulose sheets (BA 85, Schleicher & Schuell, Dassel, FRG) for 15 h at 100 V [17]. The nitrocellulose sheets were then reacted with the appropriate dilution of rabbit antiserum followed by anti-rabbit IgG alkaline phosphatase conjugate as specified by the manufacturers instructions (Promega Biotek, Madison, WI, USA).

3. RESULTS

Membranes prepared from human brain frontal lobe cortex, SK-N-SH, SH-SY5Y, SH-IN and SH-EP cells were treated with [α-^{32}P]NAD in the presence or absence of pertussis toxin. Following ADP-ribosylation in the presence of pertussis toxin, membrane proteins from human brain frontal lobe cortex show two bands of approx. 41 kDa and 39 kDa on SDS-polyacrylamide gradient gels (fig.1). The lower band ($G_{o\alpha}$) is more intensely labelled than the upper band ($G_{i\alpha}$). Membranes from SH-SY5Y cells also yield two bands of approx. 41 kDa ($G_i\alpha$) and 39 kDa ($G_{o\alpha}$) with comparable labelling intensity, whereas membranes from SK-N-SH and SH-IN cells show a higher labelling of $G_{i\alpha}$ compared to $G_{o\alpha}$. Further a third band of pertussis toxin substrate (labelled $G_{40\alpha}$) appears between these two well characterized α-subunits in SK-N-SH and SH-IN membranes. In contrast the labelled 39 kDa band is undetectable in membranes from non-neural SH-EP cells, indicating the absence of $G_{o\alpha}$.

Immunoblot analyses using rabbit polyclonal antibodies against peptides derived from α-subunits of G-proteins were carried out to characterize further the pertussis toxin substrates in membranes from human brain frontal lobe cortex and human neuroblastoma-derived cell clones. Using a panreactive antibody directed against the highly conserved GTP-binding site present on α-subunits of G-proteins [15], a strong band of approx. 39 kDa ($G_{o\alpha}$) and a weaker band of 41 kDa ($G_{i\alpha}$) can be shown in membranes from human brain frontal lobe cortex (fig.2A). In membranes from SK-N-SH, SH-SY5Y and SH-IN cells the same two bands are present, but the lower band ($G_{o\alpha}$) is weaker than the upper band which contains both $G_{i\alpha}$ and $G_{40\alpha}$. In membranes from SH-EP cells the lower 39 kDa band recognized by the $G_{\alpha common}$ antiserum is absent, confirming that $G_{o\alpha}$ is missing, as suggested by ADP-ribosylation.

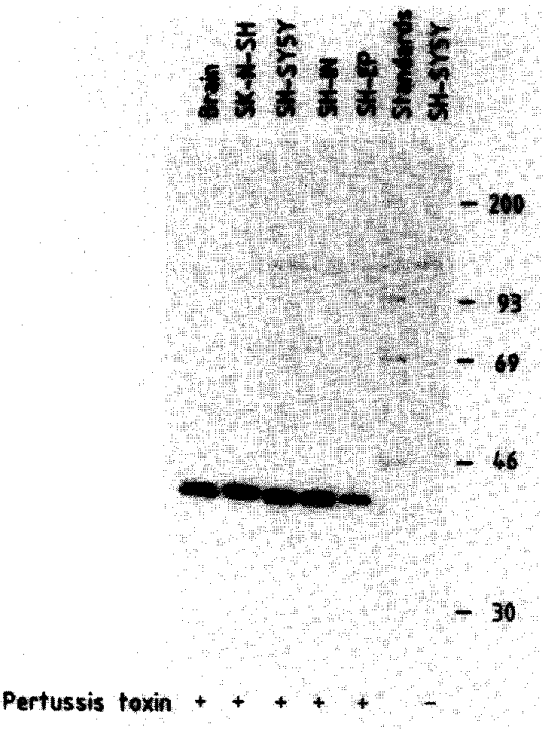

Fig.1. Pertussis toxin-dependent ADP-ribosylation of α-subunits from G-proteins in membranes from human brain frontal lobe cortex, SK-N-SH, SH-SY5Y, SH-IN and SH-EP cells. Membranes were treated with [α-^{32}P]NAD in the presence (+) or absence (–) of pertussis toxin, and subjected to SDS-PAGE and autoradiography. 10 μg membrane proteins were loaded per lane. Values on the right indicate the positions of molecular mass marker proteins kDa).

We further used antibodies directed against the α-subunit of G_o [15] to confirm the absence of $G_{o\alpha}$ in membranes from SH-EP cells. Fig.2B shows the presence of high amounts of $G_{o\alpha}$ in membranes from human brain frontal lobe cortex, intermediate amounts in membranes from SK-N-SH, SH-SY5Y and SH-IN cells and the complete absence of $G_{o\alpha}$ in membranes from SH-EP cells. The amount of $G_{o\alpha}$ per μg membrane protein is approx. 5-times higher in frontal lobe cortex of human brain than in membranes from the neuroblast clone SH-SY5Y.

Antibodies against the α-subunit of a third pertussis toxin-sensitive G-protein with an in-

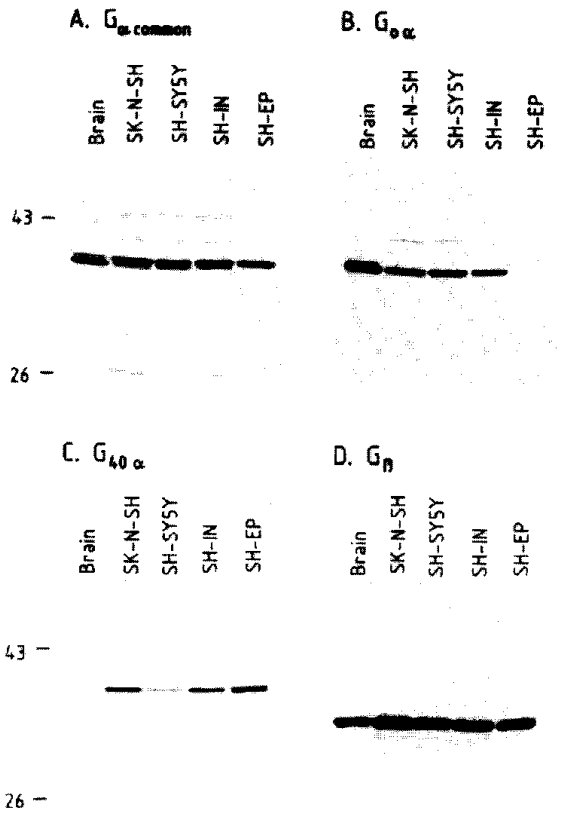

Fig.2. Immunoblot analysis of membrane proteins from human brain frontal lobe cortex, SK-N-SH, SH-SY5Y, SH-IN and SH-EP cells with antibodies specific for $G_{\alpha common}$ (A), $G_{o\alpha}$ (B), $G_{40\alpha}$ (C) and G_{β} (D). 40 μg membrane proteins from human brain frontal lobe cortex, 120 μg from SK-N-SH cells and 80 μg from other cells were subjected to SDS-PAGE, transferred to nitrocellulose and reaced with $G_{\alpha common}$ antiserum (diluted 1:5000), $G_{o\alpha}$ antiserum (diluted 1:1000), $G_{40\alpha}$ antiserum (diluted 1:1000) or G_{β} antiserum (1:2000). Values on the left indicate the position of molecular mass marker proteins (kDa).

termediate molecular mass of approx. 40 kDa [2] were raised in rabbits and were shown to cross-react with neither $G_{o\alpha}$ nor $G_{i\alpha}$ (Lang and Costa, submitted). Fig.2C shows the presence of high levels of $G_{40\alpha}$ in membranes of the cell clone SH-EP, intermediate levels in the membranes of SH-IN and SK-N-SH cells and low levels in membranes of the cell clone SH-SY5Y and human brain frontal lobe cortex. Since we have no antibody directed specifically against $G_{i\alpha}$ and the resolution power of the SDS-polyacrylamide gradient gels does not allow us to separate $G_{i\alpha}$ from $G_{40\alpha}$, we cannot determine the relative proportion of these two proteins in membranes from SH-EP cells.

Using antibodies against the β-subunit of G-proteins [15] we find that similar amounts of G_{β} per μg membrane protein are present in membranes from human brain frontal lobe cortex, SK-N-SH, SH-SY5Y, SH-IN, and SH-EP cells (fig.2D). Thus the composition of G-protein in human neuroblastoma-derived cell clones differs only in the α-subunits but not in the total amount of the common β-subunit.

Membranes prepared from SH-SY5Y cells differentiated with retinoic acid or nerve growth fac-

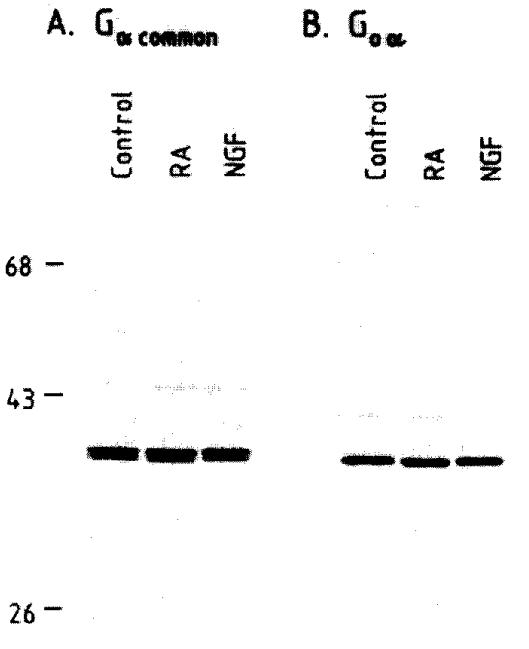

Fig.3. Immunoblot analysis of membrane proteins from SH-SY5Y cells and SH-SY5Y cells treated with retinoic acid (RA) or nerve growth factor (NGF) with antibodies specific for $G_{\alpha common}$ (A) and $G_{o\alpha}$ (B). 80 μg membrane protein per lane were subjected to SDS-PAGE, transferred to nitrocellulose and reacted with $G_{\alpha common}$ antiserum (diluted 1:5000) or $G_{o\alpha}$ antiserum (diluted 1:1000). Values on the left indicate the position of molecular mass marker proteins (kDa).

tor were analyzed by immunoblotting, using both the $G_{\alpha common}$ and $G_{o\alpha}$ antiserum. As shown in fig.3, treatment of SH-SY5Y cells with retinoic acid (RA) or nerve growth factor (NGF) does not alter the immunoreactivity of these two antibodies in membranes of differentiated cells compared to control cells.

The data presented are representative of two different batches of cells both for analysis of treated and untreated cell membranes.

4. DISCUSSION

The three subclones of the human neuroblastoma cell line SK-N-SK (SH-SY5Y, SH-IN, and SH-EP) are suitable to study the phenotypic variation within the neural crest lineage, because they are relatively stable and interconvert at a very slow rate [7]. Besides morphological differences, dramatic biochemical differences were observed for these three subclones, including expression of neurotransmitter systems and myc protooncogenes [11]. The differential expression of G-proteins could be one mechanism by which a cell can alter coupling of membrane receptors to intracellular effector systems and thereby responsiveness to extracellular signals. In the present work we have analyzed the composition of pertussis toxin sensitive G-proteins in membranes derived from three subclones of the SK-N-SH cell line and find dramatic differences between these subclones.

Membranes from the subclone SH-SY5Y with a neuroblastic phenotype show a pattern of G-protein α-subunits very similar to that of membranes from human frontal lobe cortex: high levels of $G_{o\alpha}$ and $G_{i\alpha}$ but low levels of $G_{40\alpha}$. In contrast, membranes from the neuroblastoma × glioma cell line NG 108-15 show a pattern of α-subunits from G-proteins (high concentration of $G_{40\alpha}$) which is different from that of mammalian brain cortex (unpublished). This suggests that SH-SY5Y cells provide a model of central nervous system neurons more suitable than the commonly used neuroblastoma hybrid lines.

As indicated by the pattern of G-proteins, the human neuroblastoma cell line Sk-N-SH with high levels of $G_{o\alpha}$ and $G_{i\alpha}$ and intermediate levels of $G_{40\alpha}$ represents a mixture of neuroblast-like (SH-SY5Y, SH-IN) and non-neuronal (SH-EP) cells. The neuroblast-like cell clones SH-SY5Y and SH-IN differ in the amount of $G_{40\alpha}$ present in their membranes, whereas levels of $G_{o\alpha}$ and $G_{i\alpha}$ are almost identical. The neuroblast-like cell clone SH-SY5Y and the non-neuronal clone SH-EP differ dramatically in the level of $G_{o\alpha}$ and $G_{40\alpha}$ in their membranes. While $G_{o\alpha}$ is present at high levels in SH-SY5Y cells but not detectable in SH-EP cells, $G_{40\alpha}$ is present in low levels in SH-SY5Y cells but strongly expressed in SH-EP cells. Ross et al. [7] have provided evidence that the neuronal and non-neuronal cells of SK-N-SH interconvert. Therefore during interconversion of neuroblast-like SH-SY5Y cells to non-neuronal SH-EP cells the expression of $G_{o\alpha}$ must be completely blocked whereas the expression of $G_{40\alpha}$ is strongly enhanced. These findings parallel the fact that neurotransmitter systems and the proposed oncogenes c-myc and N-myc are expressed in neuroblast-like SH-SY5Y and SH-IN cells but not in non-neuronal SH-EP cells [11].

Although we cannot provide direct evidence for a relationship between changes in expression of G-proteins and neurotransmitter systems during interconversion of these cells, it is likely that expression of neurotransmitter systems and α-subunits of G-proteins are under coordinate control that parallels observed morphological changes during interconversion. An intriguing problem is the question of which G-protein interacts with which receptor. The simultaneous expression of opioid receptors. muscarinic receptors and $G_{o\alpha}$ in the neuronal clones suggests a possible interaction. However, additional studies are needed to address this problem.

SH-SY5Y cells differentiate morphologically and biochemically in the presence of low concentrations of retinoic acid. The observed changes during differentiation include induction of neurite outgrowth, partial inhibition of cell growth, increase in neuron-specific enolase activity and increase in noradrenaline content [8]. Furthermore, differentiation of SH-SY5Y cells by retinoic acid increases the opioid receptor density by as much as two-fold and enhances opioid-mediated inhibition of adenylate cyclase [9,19]. Gierschik et al. [20] have shown that differentiation of 3T6-L1 cells from fibroblasts to adipocytes is accompanied by changes in the level of G_i and G_o. Here we show that differentiation of SH-SY5Y cells by neither

retinoic acid nor nerve growth factor leads to any change in the level of $G_{o\alpha}$ or $G_{i\alpha}$ in the membrane. Thus it appears that differentiation of cells does not necessarily involve a change in the membrane level of G-protein α-subunits. Such a change may be dependent on the cell type and/or the differentiation agent used.

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