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Equivalent regulation of wild type and an epitope-tagged variant of $G_s\alpha$ by the IP prostanoid receptor following expression in neuroblastoma × glioma hybrid, NG108-15, cells

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Abstract NG108-15 cells were transfected to stably express a haemagglutinin epitope-tagged variant of the long isoform of $G_s\alpha$. Clone BST15 expressed this polypeptide at similar levels to the endogenous long isoform of $G_s\alpha$. Treatment of clone BST15 with the IP prostanoid receptor agonist iloprost resulted in down-regulation of both forms of $G_s\alpha$ with both dose–effect curves to iloprost and time courses of loss of the two forms of $G_s\alpha$ being indistinguishable. These results demonstrate that the IP prostanoid receptor interacts with and regulates the epitope-tagged variant of $G_s\alpha$ in an equivalent manner to the unmodified protein and indicates that the epitope-tagged polypeptide can be used to analyse mechanisms of receptor regulation of cellular G-protein levels.

Key words: G-protein; Receptor; Epitope-tag; Neuroblastoma; Prostaglandin

1. Introduction

Many hormones, neurotransmitters and growth factors exert their effects on target cells and tissues by modulating the intracellular levels of various second messengers, a process mediated via members of a family of guanine nucleotide binding proteins (G-proteins) [1]. Sustained exposure of cells or tissues to agonists which function via G-protein-linked receptors frequently results in down-regulation of that receptor, attenuating cellular responses to the ligand. Receptors are not the only components of this signalling process that are regulated by ligand: the levels of cellular G-proteins are also reduced upon receptor activation in many cases [2]. Further, this down-regulation appears to be specific to that G-protein with which the receptor interacts [2].

Neuroblastoma × glioma hybrid, NG108-15, cells express endogenously an IP prostanoid receptor, activation of which results in stimulation of adenylyl cyclase. Prolonged treatment of these cells with iloprost, an agonist at this receptor results in a marked decrease in cellular levels of the α subunit of G_s without altering the levels of any other G-proteins [3–5]. We have also recently transfected NG108-15 cells to express the human β2-adrenoceptor and demonstrated that exposure of cells of clone βN22 derived from this transfection to isoprenaline also results in a specific down-regulation of $G_s\alpha$ [6]. In both CHO cells transfected to express the muscarinic M1 acetylcholine receptor [7] and in αT3-1 cells which express endogenously the receptor for gonadotrophin releasing hormone [8], down-regulation of the phosphoinositidase Cβ-linked G-proteins G_q and G_{11} occurs upon sustained exposure to agonist. In each of these cells the mechanism has been shown to be agonist-induced accelerated turnover of the G-proteins ([9] and Shah et al., unpublished observations). We have not been able to ascertain directly the mechanism of prostanoid and β-adrenoceptor-mediated down-regulation of $G_s\alpha$ in NG108-15 cells and the transfectants derived from them as available anti-$G_s\alpha$ antisera have poor immunoprecipitation properties. As a potential means to address this question herein we have transfected NG108-15 cells with an epitope-tagged version of the long isoform of $G_s\alpha$ in which a haemagglutinin epitope from the human influenza virus has been incorporated into the polypeptide (HA-$G_s\alpha$(L)) [10]. In the present study we examine the ability of agonist occupation of the IP prostanoid receptor to interact with and regulate cellular levels of HA-$G_s\alpha$(L). We demonstrate that the IP prostanoid receptor interacts with HA-$G_s\alpha$(L) and that it shows no preference for endogenous $G_s\alpha$(L) over the epitope-tagged version of the polypeptide.

2. Materials and methods

2.1. Generation and isolation of clones of NG108-15 cell variants expressing HA-$G_s\alpha$(L)

NG108-15 cells were stably co-transfected with plasmid pcDNA1 (10 μg) containing a cDNA encoding the long isoform of $G_s\alpha$ in which the haemagglutinin epitope (VPDYA) was constructed between amino acid residues 76–82 [10] (a kind gift from Drs M.J. Levis and H.R. Bourne, University of California at San Francisco, CA, USA) and plasmid pBABE hygro (1 μg) which confers resistance to hygromycin B. Clones which were resistant to hygromycin B (200 μg/ml) were selected and expanded.

2.2. Cell culture

Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) which was supplemented to 5% (v/v) with foetal bovine serum. This medium was further supplemented with hypoxanthine, aminopterin, thymidine, 100 U/ml of penicillin and 100 μg/ml streptomycin. Transfectants were routinely grown in medium containing hygromycin B (200 μg/ml).

2.3. Membrane preparation

Cells were harvested, washed twice in phosphate-buffered saline and pelleted by centrifugation at 500 × *g* for 10 min on a Beckman TJ-6 benchtop centrifuge. Membranes were prepared by homogenizing the cell pellet with 20 strokes of a Teflon/glass homogenizer in 10 mM Tris-HCl, 0.1 mM EDTA pH 7.5 (buffer A). The resultant supernatant was centrifuged at 500 × *g* for 10 min in a Beckman L5-50B centrifuge with a Ti50 rotor, to remove unbroken cells and nuclei. The supernatant was further centrifuged at 48,000 × *g* for 10 min. The pellet from this centrifugation was resuspended in buffer A and recentrifuged at

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48,000 × g for 10 min. The resultant pellet was resuspended in an appropriate volume of buffer A, aliquoted and stored at −80°C until use. Protein concentration was measured according to the method of Lowry et al. [11].

2.4. Immunoblotting

Membranes were resolved by SDS-PAGE (10% (w/v) acrylamide) and subsequently immunoblotted with a range of antisera as previously described [12]. Antiserum CS, previously characterised in [13], was raised in a New Zealand White rabbit after immunization with a glutaraldehyde conjugate of keyhole limpet haemocyanin and a synthetic peptide, RMHLRQYELL, which corresponds to the C-terminal decapeptide common to all isoforms of $G_s\alpha$. This sequence is also preserved in HA-$G_s\alpha$(L). Monoclonal antibody 12CA5 which identifies a nonapeptide sequence (YPYDVPDYA) derived from the haemagglutinin protein of human influenza virus, was purchased from Boehringer Mannheim. Immunoblots were quantitated using a Bio-Rad GS-670 Imaging Densitometer linked to an Apple Macintosh Quadra 800 microcomputer.

3. Results

NG108-15 cells were co-transfected with a cDNA encoding the long isoform of $G_s\alpha$, the stimulatory G-protein of the adenylyl cyclase cascade, into which a five amino acid sequence corresponding to an epitope derived from the haemagglutinin protein of influenza virus had been engineered between residues 76–82 [10] in plasmid pcDNA1 and the plasmid pBABE hygro to allow for selection based on resistance to hygromycin B. Resistant colonies were selected and expanded. Membranes were prepared from the clones, resolved using SDS-PAGE and immunoblotted with antiserum CS, an antipeptide antiserum raised against the C-terminal decapeptide common to all splice variants of $G_s\alpha$. Clone BST15, expressed an immunoreactive doublet (Fig. 1, panel A). The band migrating more rapidly through the gel comigrated with the 45 kDa (long) isoform of

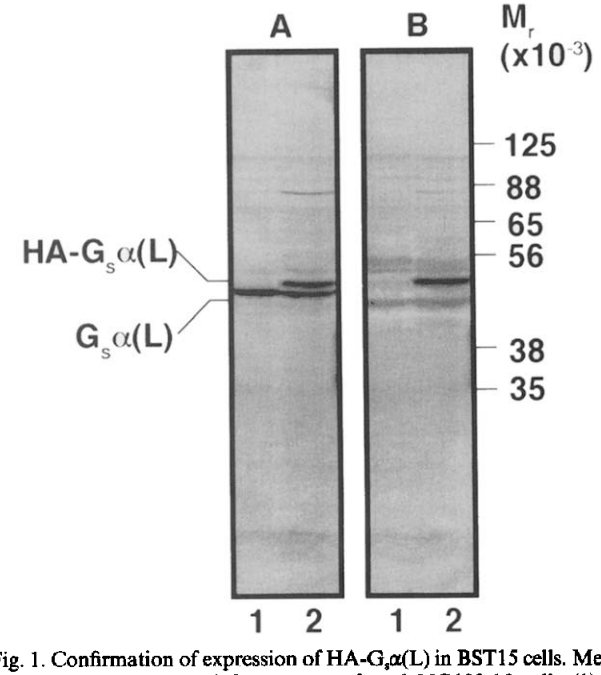

Fig. 1. Confirmation of expression of HA-$G_s\alpha$(L) in BST15 cells. Membranes (25 μg) prepared from untransfected NG108-15 cells (1) or BST15 cells (2) were resolved on SDS-PAGE (10% (w/v) acrylamide) and immunoblotted with either the $G_s\alpha$ antiserum CS (1:500 dilution) (panel A) or the HA epitope-tag antibody 12CA5 (1:400 dilution) (panel B).

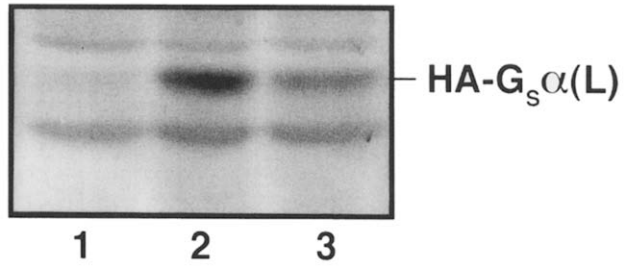

Fig. 2. The IP prostanoid receptor is able to interact with and down-regulate HA-$G_s\alpha$(L) in BST15 cells. Membranes (25 μg) prepared from NG108-15 cells (1), untreated BST15 cells (2) and iloprost-treated (1 μM, 16 h) BST15 cells (3) were resolved on SDS-PAGE (10% (w/v) acrylamide) and immunoblotted with antiserum 12CA5 (1:400 dilution) to detect HA-$G_s\alpha$(L). Iloprost treatment caused a substantial reduction in levels of immunodetectable HA-$G_s\alpha$(L) (see section 3 for details).

$G_s\alpha$ endogenously expressed by the parental cells whilst the less rapidly migrating band did not comigrate with $G_s\alpha$ immunoreactivity in the parental cells and was presumed to be the epitope-tagged version of this polypeptide, HA-$G_s\alpha$(L) (Fig. 1, panel A). Immunoblot analysis of these membranes with antiserum 12CA5, which specifically recognises the engineered epitope tag region, confirmed the upper immunoreactive species to be HA-$G_s\alpha$(L) and its expression in BST15 cells but not in parental NG108-15 cells (Fig. 1, panel B). Comparison of densitometric analyses of such immunoblots revealed that BST15 cells expressed HA-$G_s\alpha$(L) at a level of some 59.5 ± 8.8% (mean ± S.E.M.; n = 7) of the endogenous $G_s\alpha$(L) in these cells. As the parental cells express $G_s\alpha$(L) at some 9.6 pmol/mg of membrane protein [5] then the levels of HA-$G_s\alpha$(L) are some 5.7 pmol/mg membrane protein.

To determine whether HA-$G_s\alpha$(L) was able to interact with the IP prostanoid receptor, BST15 cells were incubated in the presence of the IP prostanoid agonist iloprost (1 μM, 16 h), which has been shown previously to cause a marked decrease in the cellular levels of $G_s\alpha$(L) without altering the levels of any other G-protein α subunits [3–5]. Immunoblot analysis of membranes prepared from untreated and iloprost-treated cells with either antiserum CS (see later) or antiserum 12CA5 (Fig. 2) revealed that iloprost treatment resulted in a reduction in the levels of both $G_s\alpha$(L) and HA-$G_s\alpha$(L). Densitometric scanning of a number of these immunoblots showed that the levels of $G_s\alpha$(L) were reduced to 45.1% ± 17.2 (mean ± S.E.M.; n = 5) of the levels observed in membranes from untreated cells. Similarly the levels of HA-$G_s\alpha$(L) were reduced to 48.1% ± 14.9 (mean ± S.E.M.; n = 5) of control levels in iloprost-treated cells.

Treatment of BST15 cells with different concentration of iloprost for 16 h followed by immunoblotting with antiserum CS indicated that half-maximal decrease in the levels of both HA-$G_s\alpha$(L) and $G_s\alpha$(L) was obtained with approximately 1 nM agonist (Fig. 3). Time courses of iloprost treatment (1 μM) indicated that half-maximal down-regulation was achieved within 1–2 h for both polypeptides and that new steady-state levels were produced within approximately 8 h (Fig. 4).

4. Discussion

Agonist-mediated down-regulation of G-protein α subunits

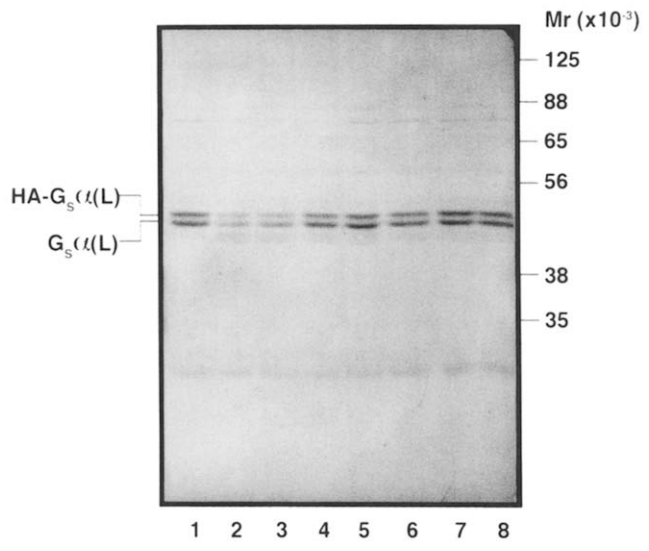

Fig. 3. Dose–response curves for iloprost-induced decrease in the cellular level of HA-$G_s\alpha$(L) and $G_s\alpha$(L) in clone BST15. Immunological detection of HA-$G_s\alpha$(L) and $G_s\alpha$(L) in membranes of BST15 clones in (1) untreated cells or after treatment with iloprost for 16 h at (2) 1 μM, (3) 100 nM, (4) 10 nM, (5) 1 nM, (6) 100 pM, (7) 10 pM, (8) 1 pM. Membranes (25 μg) prepared from these cells were resolved on SDS-PAGE (10% (w/v) acrylamide) and immunoblotted with antiserum CS (1:500 dilution).

has been observed for members of each of the G_s, G_i and G_q families of G-proteins [2]. In CHO cells transfected to express the human M1 muscarinic acetylcholine receptor, incubation with the agonist carbachol resulted in a reduction in the total cellular levels of G_q/G_{11}, the G-proteins that interact with this receptor [7]. Further, Mitchell et al. [9] were able to demonstrate that agonist treatment did not substantially alter mRNA levels of either $G_{q\alpha}$ or $G_{11\alpha}$ but did show that the half life of a substantial proportion of the total cellular levels of G_q/G_{11} was reduced from 18 h to 2.5 h upon addition of carbachol [9]. Thus, the observed reduction in the levels of the activated G-protein was primarily a consequence of agonist enhancement of turnover of the polypeptide.

Maintained agonist occupation of the IP prostanoid receptor of NG108-15 cells results in a specific down-regulation of $G_s\alpha$ [3–5]. This also occurs without substantial alterations in levels of $G_s\alpha$ mRNA levels [3] and may well also reflect accelerated protein degradation. However, the antisera we have generated (and those from a number of other workers) to $G_s\alpha$ are inefficient in immunoprecipitation assays (see [10] for discussion) and this restricts the possibility of analysing turnover of the protein directly. Levis and Bourne [10] have constructed an epitope tagged variant of $G_s\alpha$(L) (HA-$G_s\alpha$(L)) in which the sequence VPDYA was engineered into the amino acid sequence derived from exon 3, the region which is lacking in the short isoforms of $G_s\alpha$. As the bulk of evidence indicates that long and short isoforms of $G_s\alpha$ show little functional difference then it was anticipated that alterations in this region might not interfere with the function of the epitope tagged polypeptide [10]. To some extent this has been confirmed [10] but in studies which have been designed to analyse the role of post-translational palmitoylation in the membrane association of $G_s\alpha$ very different results have been obtained in studies using the epitope tagged form of HA-$G_s\alpha$(L) [14] from those using the unmodified sequence [15,16]. While other explanations, such as the cell lines used, may contribute to these observed differences they clearly indicate that considerable analysis must be performed to ensure that HA-$G_s\alpha$(L) is suitable for any envisaged set of studies, particularly in this case as agonists stimulate depalmitoylation of $G_s\alpha$ [17–19] and this may be an early step in agonist-induced down-regulation of the polypeptide.

Thus, in the present study we have stably transfected NG108-15 derived cells to express HA-$G_s\alpha$(L) and subsequently compared in parallel the ability of IP prostanoid receptor activation to regulate both endogenous $G_s\alpha$(L) and the introduced HA-$G_s\alpha$(L). Clone BST-15 was selected for detailed analysis as it expressed relatively similar levels of $G_s\alpha$(L) and HA-$G_s\alpha$(L) (Fig. 1). We were fortunate in these studies that HA-$G_s\alpha$(L) migrates in SDS-PAGE somewhat more slowly than $G_s\alpha$(L) (Figs. 1 and 3) which allowed for easy concurrent detection and analysis of the two polypeptides.

Treatment of BST15 cells with iloprost not only resulted in a substantial decrease in the levels of endogenous $G_s\alpha$(L) but also in the levels of HA-$G_s\alpha$(L), indicating that the IP prostanoid receptor was able to interact with the epitope-tagged G-protein (Fig. 2). Further, the degree of receptor-G-protein interaction was similar for $G_s\alpha$(L) and HA-$G_s\alpha$(L) as assessed by the similar agonist concentrations needed to achieve half-maximal G-protein down-regulation (Fig. 3). Agonist-promoted removal of both $G_s\alpha$(L) and HA-$G_s\alpha$(L) from the cell was a rapid process with half-maximal loss for both forms occurring within approximately 1–2 h (Fig. 4).

The demonstration that HA-$G_s\alpha$(L) levels are regulated in a manner indistinguishable from the endogenously expressed $G_s\alpha$(L) indicates that this construct may be used to analyse in

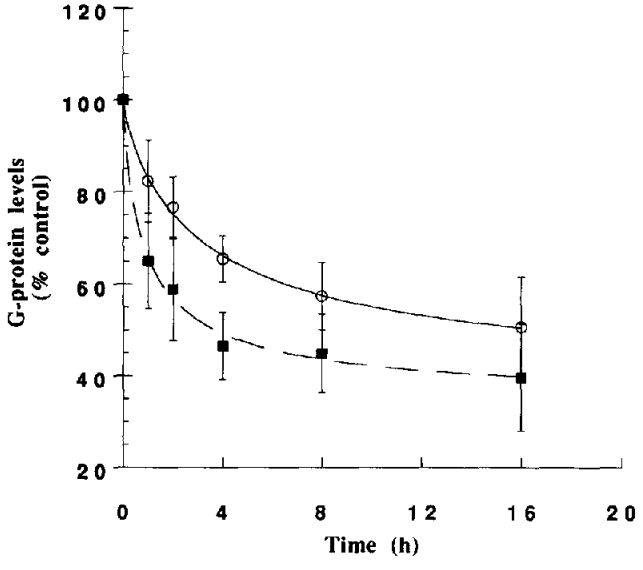

Fig. 4. Concurrent down-regulation of HA-$G_s\alpha$(L) and $G_s\alpha$(L) by iloprost in clone BST15. After treatment with 1 μM iloprost for various times, the levels of immunologically detected $G_s\alpha$(L) and HA-$G_s\alpha$(L) in membranes from BST15 clones were assessed. Membranes (25 μg) were resolved on SDS-PAGE (10% (w/v) acrylamide) and immunoblotted with antiserum CS (1:500 dilution). A series of such immunoblots were subjected to quantitation by densitometric scanning. Levels of $G_s\alpha$(L) (open symbols) and HA-$G_s\alpha$(L) (closed symbols) are presented as a % ± S.E.M. of those from untreated cells, $n = 6$. Apart from the 4 h time point the data did not display statistically significant differences between the extent of $G_s\alpha$(L) and HA-$G_s\alpha$(L) down-regulation.

detail the mechanisms of G-protein loss. The use of antisera directed against epitope-tagged G-proteins will potentially also be useful in systems where there are no G-protein antisera available.

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