Interaction of the G-protein $G_{11}\alpha$ with receptors and phosphoinositidase C:

The contribution of G-protein palmitoylation and membrane association

Alan Wise[a], Marco Parenti[b], Graeme Milligan[a,*]

[a] *Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, UK*

[b] *Dipartimento di Pharmacologia, Università di Milano, Via Vanvitelli 32, 20129 Milan, Italy*

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Abstract Wild-type and palmitoylation defective mutants of the murine G protein $G_{11}\alpha$ were transfected into HEK293 cells. Wild-type $G_{11}\alpha$ was membrane associated, Cys9Ser Cys10Ser $G_{11}\alpha$ was present in the soluble fraction whilst both Cys9Ser $G_{11}\alpha$ and Cys10Ser $G_{11}\alpha$ were distributed between the fractions. Expression of the rat TRH receptor resulted in agonist stimulation of inositol phosphate accumulation. The degree of stimulation produced by TRH following co-transfection of the palmitoylation-resistant forms of $G_{11}\alpha$ compared to the wild-type protein correlated with the amount of membrane-associated G protein.

Key words: G protein; Acylation; Receptor; Phospholipase C; Inositol

1. Introduction

The phosphoinositidase C-linked G proteins, $G_q\alpha$ and $G_{11}\alpha$ can be post-translationally acylated on adjacent cysteine residues at position 9 and 10 [1–3]. Previous studies on acylation-resistant mutants of $G_q\alpha$ have indicated both that they are either entirely or partially cytosolic [1] or that the mutations did not have a significant effect on particulate versus cytosolic partitioning [3,4]. However, particulate Cys9Ala Cys10Ala $G_q\alpha$ has been reported to be resistant to solubilisation by sodium cholate, the detergent routinely used in the early stages of G-protein purification [4], which may indicate it to represent an incorrectly folded or denatured protein.

We have recently noted that a rodent version of $G_{11}\alpha$ can be effectively resolved from primate versions of the same G protein in urea-containing SDS-PAGE systems [5,6] and have taken advantage of this fact to generate a stable cell line derived from (human) HEK293 cells which expresses both human and murine versions of this G protein. Concomitant activation of both the human and murine versions of this G protein could then be shown to be produced by expression of the long isoform of the rat thyrotropin-releasing hormone (TRH) receptor [6].

In the current study we utilise the capacity to unambiguously discriminate human and murine forms of $G_{11}\alpha$ to examine the role of potential post-translational acylation and cellular location on interactions of the G protein with both the rat TRH receptor and phosphoinositidase C.

2. Materials and methods

2.1. Materials

[^{3}H]Inositol was from Amersham International. Lipofectamine and all cell culture reagents were from Life Technologies (Paisley, UK). Nitrocellulose was from Costar (Cambridge, MA). All other reagents were from Fisons and were of the highest grade commercially available.

2.2. Generation of Cys9Ser, Cys10Ser and Cys9Ser Cys10Ser mutants of murine $G_{11}\alpha$

Murine $G_{11}\alpha$ in the mammalian expression vector pCMV was a kind gift from Dr. M.I. Simon, California Institute of Technology, California, USA. The Cys9Ser, Cys10Ser and Cys9Ser Cys10Ser mutations were generated as in [3] except that they were subcloned into pcDNA3 (Invitrogen) for transfection.

2.3. Maintenance and transfection of HEK293 cells

HEK293 cells were maintained in DMEM supplemented with 10% (v/v) newborn calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37°C. Cells were transfected at approximately 40% confluency with pcDNA3 containing either the long isoform of the rat TRH receptor [6,7] in isolation or in combination with wild-type, Cys9Ser, Cys10Ser or Cys9Ser Cys10Ser murine $G_{11}\alpha$ using Lipofectamine reagent (Life Technologies, Paisley, UK) according to the manufacturers' instructions.

2.4. Inositol phosphate measurements

Twenty-four hours after transfection the cells were reseeded into 12-well plates and allowed to grow for a further 24 h. Cells were then labelled with [^{3}H]inositol (1 μCi/ml) and 24 h later total [^{3}H]inositol phosphate production was measured either basally or upon addition of TRH (10 μM) as in [6].

2.5. Sodium cholate extractions

Cells were harvested, freeze/thawed, resuspended in 400 μl of Tris/EDTA and homogenised with a Teflon/glass homogenizer. Following a low-speed centrifugation, to remove unbroken cells and the nuclear pellet (P1), the samples were centrifuged at 200 000 × *g* for 30 min at 4°C and the soluble cytoplasmic fraction (C) retained. The particulate fraction (P2) was resuspended in a final volume of 500 μl of extraction buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 150 mM NaCl containing 1% (w/v) sodium cholate). The samples were incubated at 4°C with stirring for 1 h then centrifuged at 200 000 × *g* for 30 min at 4°C generating a soluble supernatant (S) and insoluble pellet (P).

2.6. Electrophoretic separation of species variants of $G_{11}\alpha$

Particulate, sodium cholate solubilised and cytosolic fractions were electrophoresed by SDS-PAGE in 10% (w/v) acrylamide gels containing 6 M urea. These conditions separate murine $G_{11}\alpha$ from simian and human $G_{11}\alpha$ and $G_q\alpha$ [5,6]. Proteins were transferred to nitro-

*Corresponding author. Davidson Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK. Fax: (44) 141-330-4620.
E-mail: GBCA32@UDCF.GLA.AC.UK

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cellulose and probed with antiserum CQ which recognises the C-terminal decapeptide of $G_{11}\alpha$ and $G_q\alpha$ which is entirely conserved in all of these species and visualised as described [8]. Following development, blots were scanned and quantitated on a Bio-Rad GS670 imaging densitometer (Bio-Rad, Hemel Hempstead, UK) linked to an Apple Macintosh Quadra 800 computer.

3. Results

The endogenously expressed forms of the human phosphoinositidase C-linked G-proteins $G_q\alpha$ and $G_{11}\alpha$ in HEK293 cells co-migrated in 6 M urea-containing SDS-PAGE and could be detected using an antiserum [8] directed against the C-terminal decapeptide which is entirely conserved in these two G proteins [9]. These proteins were present exclusively in the P2 particulate fraction and were completely solubilised (S) from this membrane fraction by treatment with sodium cholate (1%, 1 h, 4°C) (Fig. 1, lanes 1–4). Following transient expression of wild-type murine $G_{11}\alpha$ in HEK293 cells, this polypeptide could be detected following urea-containing SDS-PAGE as a more rapidly migrating polypeptide than the human form. Fractions of this polypeptide could be detected in both the cytosolic as well as the P2 particulate fraction but as with the endogenous human $G_{11}\alpha/G_q\alpha$ essentially all of the P2 particulate murine $G_{11}\alpha$ was solubilised from this fraction by treatment with sodium cholate (Fig. 1, lanes 5–8). Expression of either Cys9Ser or Cys10Ser mutations of murine $G_{11}\alpha$ altered the cellular distribution profile of the G protein with greater fractions of the expressed polypeptides now being located in the cytosolic fractions. However, the majority of the P2 particulate Cys9Ser and Cys10Ser murine $G_{11}\alpha$ was still solubilised by sodium cholate treatment (Fig. 1, lanes 9–16). Expression of a Cys9Ser Cys10Ser double mutation of murine $G_{11}\alpha$ resulted in the vast majority of the immunodetected protein being cytosolic and poor sodium cholate solubilisation of the small amount of P2 particulate located G protein (Fig. 1, lanes 17–20). However, as anticipated, all of the endogenous human $G_{11}\alpha/G_q\alpha$ was both present in the P2 particulate fractions of cells transfected to express the various murine $G_{11}\alpha$ mutations and was solubilised by sodium cholate (Fig. 1).

We have previously shown that Cys^9 and Cys^{10} are the sites for post-translational palmitoylation of murine $G_{11}\alpha$ and that mutation of both Cys residues to Ser entirely prevents palmitoylation of the expressed protein whilst mutation of either Cys^9 or Cys^{10} results in a decline in the palmitoylation capacity of the expressed proteins to below 20% of the wild-type protein [2]. We thus assessed the capacity of the expressed wild-type and palmitoylation-resistant forms of $G_{11}\alpha$ to mediate activation of a phosphoinositidase C. A mixture of aluminum chloride (30 μM) and sodium fluoride (10 mM) (to generate fluoroaluminate ions) was added to either mock transfected HEK293 cells or those transfected with wild-type murine $G_{11}\alpha$ or the Cys9Ser, Cys10Ser or Cys9Ser Cys10Ser forms of this G protein and which had subsequently been prelabelled with [^{3}H]inositol for 24 h. A small stimulation of [^{3}H]inositol phosphate generation was observed in the mock transfected cells (Fig. 2) presumably due to activation of the endogenous human $G_{11}\alpha/G_q\alpha$. A much more robust stimulation in response to fluoroaluminate was observed following expression of wild-type murine $G_{11}\alpha$ (Fig. 2) whereas expression of each of the palmitoylation defective mutants resulted in a considerably more limited stimulation of [^{3}H]inositol

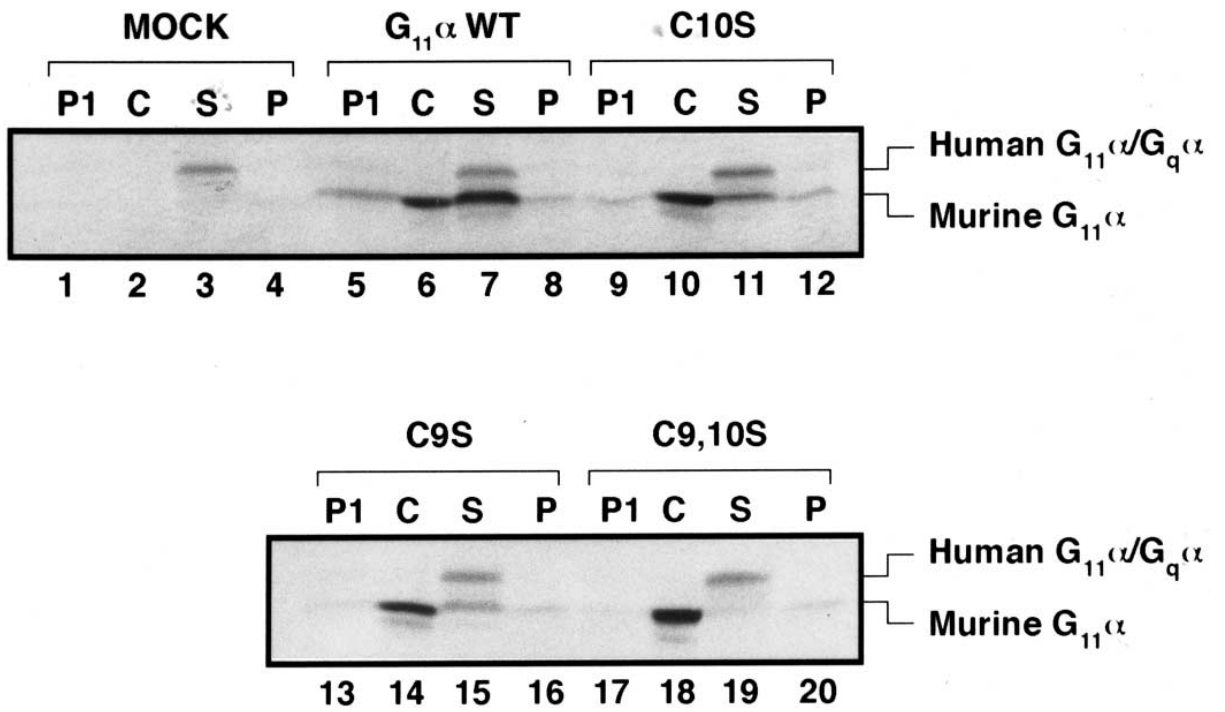

Fig. 1. Subcellular localisation and sodium cholate solubilisation of particulate wild-type and palmitoylation negative mutant forms of murine $G_{11}\alpha$ after transient expression in HEK293 cells. HEK293 cells were either mock transfected (1–4) or transfected with cDNAs encoding wild-type (5–8), Cys10Ser (9–12) Cys9Ser (13–16) or Cys9Ser Cys10Ser murine $G_{11}\alpha$ (17–20). Cells were then homogenised and separated into P1 particulate (P1) (1,5,9,13,17), cytosolic (C) (2,6,10,14,18) or P2 particulate fractions. The P2 particulate fractions were extracted with sodium cholate and separated into solubilised (S) (3,7,11,15,19) or insoluble particulate (P) (4,8,12,16,20) fractions. All samples were resolved by SDS-PAGE containing 6 M urea, transferred to nitrocellulose and immunoblotted using antiserum CQ [8].

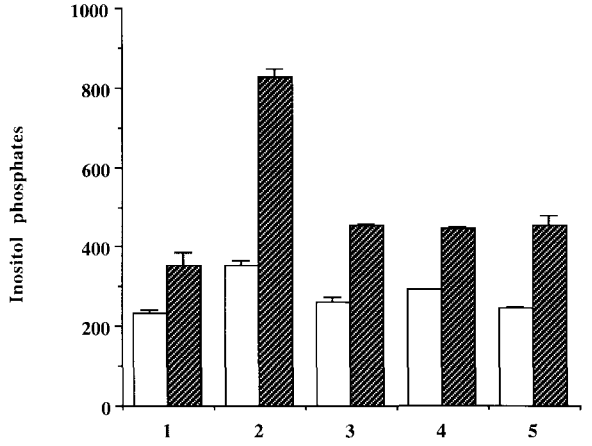

Fig. 2. The effect of fluoroaluminate and $G_{11}\alpha$ palmitoylation potential on phosphoinositidase C activity. HEK293 cells were either mock transfected (1) or transfected with cDNAs (1 μg) encoding wild-type $G_{11}\alpha$ (2), Cys9Ser $G_{11}\alpha$ (3), Cys10Ser $G_{11}\alpha$ (4) or Cys9Ser, Cys10Ser $G_{11}\alpha$ (5) in pcDNA3 as described in Section 2. Twenty-four hours after transfection the cells were reseeded into 12-well plates and allowed to grow for a further 24 h. Cells were then labelled with [^{3}H]inositol (1 μCi/ml) and 24 h later total [^{3}H]inositol phosphate production was measured either basally (open bars) or upon addition of fluoroaluminate (10 mM NaF+30 μM $AlCl_3$) (filled bars). Data are presented as total [^{3}H]inositol phosphates generated/10 000 dpm in [^{3}H]inositol containing lipids and represent means ± SEM from triplicate assays derived from one of three similar experiments performed.

phosphate production compared to that achieved with the wild-type G protein (Fig. 2).

We have previously demonstrated that stable expression of the long isoform of the rat TRH receptor in HEK293 cells allows substantial stimulation of inositol phosphate production in response to addition of TRH [10]. Transient transfection of HEK293 cells with 0.2 μg of this TRH receptor cDNA resulted in a very modest stimulation of [^{3}H]inositol phosphate production from [^{3}H]inositol prelabelled cells (Fig. 3). This could be increased by transfection of a higher level of TRH receptor cDNA (Fig. 3). At both levels of TRH receptor cDNA greater [^{3}H]inositol phosphate production was achieved by co-expression of wild-type murine $G_{11}\alpha$ (Fig. 3).

In contrast to the capacity of wild-type murine $G_{11}\alpha$ to promote TRH-induced [^{3}H]inositol phosphate production upon co-expression with the TRH receptor the capacity of the Cys9Ser and Cys10Ser mutations of $G_{11}\alpha$ was very limited (Fig. 4) and no stimulation above that observed in TRH receptor only transfected HEK293 cells was observed following co-expression of the TRH receptor and Cys9Ser Cys10Ser $G_{11}\alpha$ (Fig. 4).

4. Discussion

Post-translational acylation of cysteine residues close to the N-terminus occurs in all G-protein α-subunits with the exception of the transducins of the visual system [11–13]. In the cases of the pertussis toxin-insensitive, phosphoinositidase C-linked G proteins $G_q\alpha$ and $G_{11}\alpha$ attachment of palmitate (and potentially other acyl groups) can occur on adjacent cysteines at positions 9 and 10 [1–3]. All previous studies on the potential role of post-translational acylation have utilised $G_q\alpha$. Although it is generally assumed that $G_q\alpha$ and $G_{11}\alpha$ can function interchangeably there is no evidence that this is so in respect of their acylation properties.

Both $G_q\alpha$ and $G_{11}\alpha$ are very widely expressed [9] and this raises difficulties in examining the cellular distribution and function of mutant forms of these proteins without substantial overexpression beyond normal levels. We have recently found an alternative to this accompanying our observations that primate and rodent forms of $G_{11}\alpha$ migrate differently in urea-containing SDS-PAGE even though they are highly similar in primary amino acid sequence [5,6]. In the current experiments we have taken advantage of the endogenous expression of human $G_{11}\alpha$ in HEK293 cells by examining the cellular distribution and detergent solubility of this protein when compared to transiently introduced wild-type and acylation-resistant mutants of murine $G_{11}\alpha$.

Because of our previous observations that immunoreactive transiently expressed G protein present in the particulate pellet following low-speed centrifugation of hypotonically lysed cells largely represents improperly folded or denatured protein [2] we did not use these fractions for further analysis. All of the endogenous human $G_{11}\alpha/G_q\alpha$ was present in all experiments in the high-speed particulate fraction (P2) and could be solubilised from this by treatment with sodium cholate (Fig. 1). These observations provided a reference point for all studies on both wild-type and mutated forms of murine $G_{11}\alpha$. Essentially all of the particulate wild-type murine $G_{11}\alpha$ could be extracted with sodium cholate (Fig. 1). The percentage of P2 particulate associated forms of murine $G_{11}\alpha$ varied with the mutation introduced, with replacement of both Cys^9 and Cys^{10} by Ser resulting in only a fraction of the protein being membrane associated although the bulk of this material could be extracted with sodium cholate. By contrast, the expressed Cys9Ser Cys10Ser was almost entirely cytosolic. However,

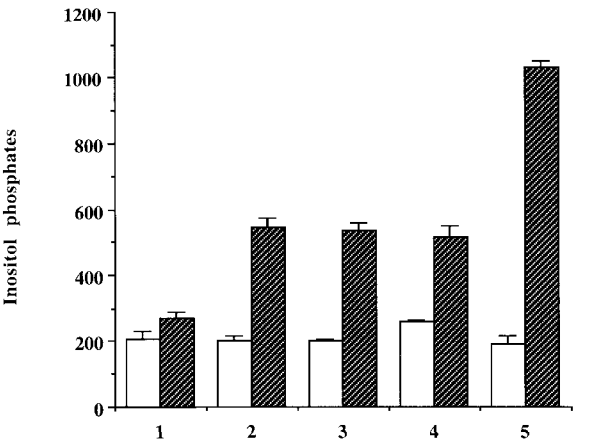

Fig. 3. Expression of the TRH receptor results in activation of phosphoinositidase C by co-expressed murine $G_{11}\alpha$. HEK293 cells were transfected with cDNAs encoding the long isoform of the rat TRH receptor (0.2 μg (1–3) or 1.0 μg (4–5)) without (1,4) or with wild-type murine $G_{11}\alpha$ (0.8 μg (2), 1.0 μg (5) or 1.8 μg (3)) in pcDNA3 (to maintain the total cDNA added per transfection at 2.0 μg) as described in Section 2. Twenty-four hours after transfection the cells were reseeded into 12-well plates and allowed to grow for a further 24 h. Cells were then labelled with [^{3}H]inositol (1 μCi/ml) and 24 h later total [^{3}H]inositol phosphate production was measured either basally (open bars) or upon addition of TRH (10 μM) (filled bars). Data are presented as total [^{3}H]inositol phosphates generated/10 000 dpm in [^{3}H]inositol containing lipids and represent means ± SEM from triplicate assays derived from one of three similar experiments performed.

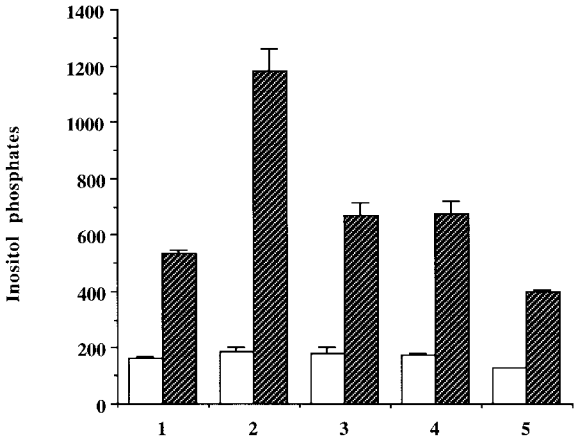

Fig. 4. Activation of phosphoinositidase C by the TRH receptor: effects of the palmitoylation potential of murine $G_{11}\alpha$. HEK293 cells were transfected with cDNAs encoding the long isoform of the rat TRH receptor (1 μg) without (1) or in combination with wild-type $G_{11}\alpha$ (2), Cys9Ser $G_{11}\alpha$ (3), Cys10Ser $G_{11}\alpha$ (4) or Cys9Ser Cys10Ser $G_{11}\alpha$ (5) (all at 1 μg) in pcDNA3 as described in Section 2. Twenty-four hours after transfection the cells were reseeded into 12-well plates and allowed to grow for a further 24 h. Cells were then labelled with [^{3}H]inositol (1 μCi/ml) and 24 h later total [^{3}H]inositol phosphate production was measured either basally (open bars) or upon addition of TRH (10 μM) (filled bars). Data are presented as total [^{3}H]inositol phosphates generated/10 000 dpm in [^{3}H]inositol containing lipids and represent means ± SEM from triplicate assays derived from one of three similar experiments performed.

although wild-type murine $G_{11}\alpha$ was strongly activated by fluoroaluminate (which mimics the terminal (γ) phosphate of GTP and thus activates the G protein) to stimulate phosphoinositidase C each of the three Cys to Ser mutants also allowed a significant, if limited, degree of stimulation of phosphoinositidase C, which was not different between the two single mutations and the double mutation. Although Edgerton et al. [4] reported weak stimulation of a phospholipase C by Cys9Ala Cys10Ala $G_q\alpha$ they did not test the two equivalent single mutants and Hepler et al. [3] have noted poor stimulation of phospholipase Cβ1 by a Cys9Ser Cys10Ser double mutant of $G_q\alpha$ whereas the two single mutants activated normally. Our results thus vary from both of these reports.

The rat TRH receptor in HEK293 cells can interact with stably co-expressed human and murine $G_{11}\alpha$ [6]. Transient transfection of this receptor into HEK293 cells allowed agonist stimulation of phosphoinositidase C activity and co-expression of the receptor along with wild-type murine $G_{11}\alpha$ resulted in substantially greater activity (Fig. 3). TRH stimulation of phosphoinositidase C beyond that due to endogenous $G_{11}\alpha/G_q\alpha$ was also observed following co-transfection with both murine Cys9Ser $G_{11}\alpha$ and Cys10Ser $G_{11}\alpha$ but not with the double mutant (Fig. 4). The degree of stimulation by TRH was correlated with the level of the P2 particulate murine $G_{11}\alpha$ forms and with their extraction by sodium cholate. As noted above, both Cys9Ser and Cys10Ser $G_q\alpha$ have been reported to be activated by receptor agonist (although to a lower level than the wild-type protein) with the double mutant showing little agonist dependent function [1]. As interaction with a receptor must be dependent upon physical location at or close to the plasma membrane the lack of ability of the cytosolic double Cys–Ser mutant $G_{11}\alpha$ to be stimulated by the TRH receptor was anticipated. The capacity of acylation resistant forms of phosphoinositidase C-linked G proteins to produce a small degree of stimulation of the effector enzyme following addition of fluoroaluminate (Fig. 2) may reflect both the cellular location of the effector and the suggestion that agonist-induced deacylation and cellular translocation of G-protein α-subunits from the membrane may occur upon activation [14,15]. However the weak stimulation compared to that produced by wild-type $G_{11}\alpha$ demonstrates that the acylation deficient mutants are functionally severely compromised. As such, membrane attachment and the potential for acylation contributes to, but may not be an absolute requirement, for direct G-protein regulation of phosphoinositidase C but is required to allow regulation by receptor agonists.

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