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Short communication

p600 STABILIZES MICROTUBULES TO PREVENT THE AGGREGATION OF CaMKIIα DURING PHOTOCONDUCTIVE STIMULATION

CAMILLE BELZIL[1], TIM RAMOS[1], KAMON SANADA[2], MICHAEL A. COLICOS[1] and MINH DANG NGUYEN[1,*]

[1]Hotchkiss Brain Institute, University of Calgary, Departments of Clinical Neurosciences, Cell Biology & Anatomy, Biochemistry & Molecular Biology, HMRB 153, 3330 Hospital Drive NW, Calgary, Alberta, Canada, T2N 4N1,
[2]Molecular Genetics Research Laboratory, Graduate School of Science, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan

Abstract: The large microtubule-associated/Ca^{2+}-signalling protein p600 (also known as UBR4) is required for hippocampal neuronal survival upon Ca^{2+} dyshomeostasis induced by glutamate treatment. During this process, p600 prevents aggregation of the Ca^{2+}/calmodulin-dependent kinase IIα (CaMKIIα), a proxy of neuronal death, via direct binding to calmodulin in a microtubule-independent manner. Using photoconductive stimulation coupled with live imaging of single neurons, we identified a distinct mechanism of prevention of CaMKIIα aggregation by p600. Upon direct depolarization, CaMKIIα translocates to microtubules. In the absence of p600, this translocation is interrupted in favour of a sustained self-aggregation that is prevented by the microtubule-stabilizing drug paclitaxel. Thus, during photoconductive stimulation, p600 prevents the aggregation of CaMKIIα by stabilizing microtubules. The effectiveness of this stabilization for preventing CaMKIIα aggregation during direct depolarization but not during glutamate treatment suggests a model wherein p600 has two modes of action depending on the source of cytosolic Ca^{2+}.

Keywords: P600, UBR4, Ca^{2+}, Ca^{2+}/calmodulin-dependent kinase IIα, Microtubules, Paclitaxel

* Author for correspondence. Email: mdnguyen@ucalgary.ca; phone: 403 2105494; fax: 403-210-8840

Abbreviations used: CaM – calmodulin, CaMKII – Ca^{2+}/calmodulin-dependent kinase II, GFP – green fluorescent protein, NMDA – N-methyl-D-aspartate, AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, DMSO – dimethyl sulfoxide

INTRODUCTION

Ca^{2+}/calmodulin-dependent kinase II (CaMKII) accounts for nearly 1% of the total protein in the mammalian brain [1]. By virtue of its toroidal arrangement, its subunits are able to phosphorylate one another, entering into a sustained period of kinase activity. This molecular hysteresis and accompanying signalling activity has led to the hypothesis that CaMKII is a medium of Ca^{2+}-dependent memory [2]. The localization and activity of CaMKII is tightly linked to Ca^{2+}-mediated neuronal survival. Under ischemic conditions *in vivo*, CaMKII rapidly enters a particulate state wherein it loses kinase activity for a prolonged period [3–5]. Experimental reproductions of the lowered pH, uncoupled oxidative phosphorylation, and Ca^{2+} overload characteristic of the ischemic brain induce the α isoform of CaMKII (CaMKIIα) to form similar long-lasting self-aggregates [6–10]. While these various reports of pathological inactivation and self-aggregation are likely to be observations of the same phenomenon, this is still uncertain, as is the role of this pathological behaviour. Furthermore, not only does CaMKII respond to Ca^{2+} influx, its activation state also controls it: CaMKII inhibition causes sufficient glutamate-induced Ca^{2+} entry to cause neuronal death under ambient culture activity [11]. Thus, studies of CaMKII, with its extraordinarily high neuronal expression, its role in synaptic plasticity and memory, and its direct regulation of neuronal survival, are of paramount importance as proxies of neuronal well-being and outcome.

p600 (or UBR4) is a large multifunctional protein (600 kDa) that plays an important role in neurite outgrowth, neuronal migration, protein degradation, Ca^{2+} signalling, cell adhesion, cell survival, and autophagy [12–16]. p600 is enriched in the brain, particularly in neurons, and has at least two microtubule-stabilizing domains [12].

We recently reported that depletion of p600 by RNAi significantly increases the proportion of neurons showing CaMKIIα aggregation upon glutamate-induced Ca^{2+} entry in hippocampal cultured neurons [17]. p600 was found to form a complex with CaM and CaMKIIα, mediated by a direct and atypical interaction between p600 and CaM. Specific disruption of this interaction resulted in neuronal death under ambient activity, and potentiated CaMKIIα aggregation following the application of mild doses of exogenous glutamate. Using an array of inhibitors, it was determined that the primary contributors to CaMKIIα aggregation were Ca^{2+} influx through N-methyl-D-aspartate receptors (NMDA) and metabotropic release of Ca^{2+} through inositol 1,4,5-trisphosphate receptors.

Importantly, pre-incubation with the CaMKII inhibitors KN62 or KN93 failed to prevent the aggregation of CaMKIIα, demonstrating that the aggregation was CaM-independent and distinct from the translocation to synapses [18]. Furthermore, stabilization of microtubules with paclitaxel had no effect, demonstrating that p600 did not prevent CaMKIIα aggregation via its microtubule-stabilizing function. Intriguingly, inhibition of voltage-gated L-type Ca^{2+} channels modestly rescued the aggregation of CaMKIIα. This suggested

that p600 could modulate CaMKIIα aggregation upon direct depolarization. To further investigate this finding, we analyzed the spatio-temporal dynamics of CaMKIIα aggregation induced by photoconductive stimulation, a technology that allows for the direct depolarization of single neurons coupled with live imaging.

MATERIALS AND METHODS

Cell culture, transfection, and photoconductive stimulation

Dissociated cultures of rat primary hippocampal neurons were prepared on silicon wafers for photoconductive stimulation as described previously [19, 20]. Neurons were co-transfected with GFP-CaMKIIα and either control or p600 RNAi vectors (molar ratio 1:2) at DIV10 [17]. The day following transfection, the silicon wafers were mounted in a specialized photoconductive stimulation apparatus [20] and immersed in a pre-warmed bath solution consisting of 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM d-glucose, and 10 mM HEPES (pH 7.3). For trials with paclitaxel, neurons were pre-incubated for 15 min with 100 nM paclitaxel (Sigma) or a DMSO vehicle (1:10,000). Individual neurons that expressed GFP-CaMKIIα were selected. Under constant light intensity, the neurons were depolarized with a 4 V square wave at 100 Hz, 5 ms pulse duration. High-resolution images were captured at ~1.4 frames per second, using a 100x water-immersion objective on an Olympus BX61WI microscope and a Watec N102 integrating CCD camera.

Quantification, curve fitting and statistical analysis

Neurons were depolarized and 5 min later categorized as displaying GFP-CaMKIIα aggregation or not (Fig. 1). Neurons that did not show GFP-CaMKIIα aggregation after the initial depolarization were depolarized for 30 s to validate the experimental setup, and those that did not show GFP-CaMKIIα aggregation after that were not used for curve fitting or statistical analysis. The data presented in Fig. 1 are fit to a Boltzmann distribution. For accurate statistical analysis, the data were treated as discrete. The mean and standard error were determined by iteratively fitting a cumulative mass function to the data. The results were used to compare the datasets using Student's t test.

Quantification of single-cell aggregation

Using ImageJ, the degree of aggregation of GFP-CaMKIIα was determined in each frame of the video by taking intensity thresholds. The automatic particle analyzing function was then used to count the average number of particles. Thirty eight different intensity thresholds were used, covering the range of intensities over which the image of the soma was not saturated. The results are plotted in the graph in Fig. 2C.

Dendrite analysis

In Fig. 2D, the images are brightness and contrast adjusted to clearly show the localization of the aggregates. The trace in the final panel was created using the

threshold-adjusting function of ImageJ, wherein the threshold was adjusted to show the initial synaptic distribution of GFP-CaMKIIα. The outline was then automatically traced and overlaid on the final frame for comparison.

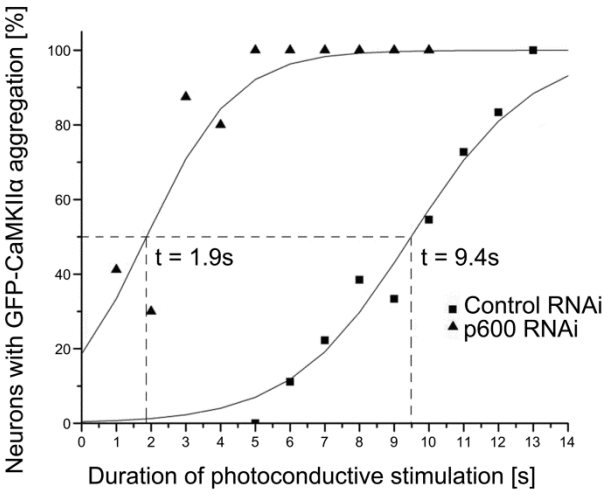

Fig. 1. Depletion of p600 reduces the length of depolarization required to cause neurons to enter a state wherein GFP-CaMKIIα is aggregated. Neurons were depolarized for the indicated time, allowed 5 min to respond, and scored for the presence or absence of GFP-CaMKIIα aggregation. When fit to a Boltzmann distribution and compared against one another, the data show that depletion of p600 by RNAi causes a mean 80% reduction in the length of depolarization required to cause GFP-CaMKIIα to aggregate (control RNAi, n = 78 neurons; p600 RNAi, n = 50 neurons; $t_3 = 17.7$, $p < 0.001$ by Student's t test).

RESULTS

Depletion of p600 lowers the threshold of GFP-CaMKIIα aggregation following photoconductive stimulation

Our previous findings showed that inhibiting L-type Ca^{2+} channels had a significant effect in preventing CaMKIIα aggregation following the bath application of glutamate and glycine [17]. Based on that, we assessed if direct depolarization of single neurons could cause CaMKIIα aggregation. We constructed a dose-response curve wherein single neurons were depolarized by photoconductive stimulation [19, 20] for a given time, incubated for an additional 5 min, then scored for the presence or absence of GFP-CaMKIIα aggregates.

When transfected with a control RNAi, neurons formed aggregates after an average of 9.49 ± 0.24 s (± SEM, n = 78) of depolarization. We then assessed if knockdown of p600 would potentiate this effect. When p600 is knocked down by RNAi, neurons form aggregates after an average of only 1.77 ± 0.36 s (n = 50), resulting in a left-shifted dose-response curve ($t_3 = 17.7$, $p < 0.001$ by Student's t test). Thus, the absence of p600 predisposes neurons to undergo CaMKIIα aggregation after direct depolarization.

Depletion of p600 interrupts the cytoskeletal localization of GFP-CaMKIIα with irreversible aggregation

A recent study showed that during KCl depolarization, CaMKIIα undergoes a rapid and sustained translocation to microtubules [21]. Since the microtubule translocation and self-aggregation of CaMKIIα can both be caused by depolarization, but are mutually exclusive behaviours, we sought to compare the time-course and localization of GFP-CaMKIIα during neuronal depolarization. We co-transfected single neurons with GFP-CaMKIIα and control or p600 RNAi, depolarized them at 0 s (for 5 s), and filmed for 5 min.

We live-imaged 9 control RNAi-transfected neurons and 11 p600 RNAi-transfected neurons. Fig. 2 shows representative examples. Fig. 2A and Suppl. movies 1 and 2 in Supplementary material at http://dx.doi.org/10.2478/s11658-014-0201-9 depict a neuron co-transfected with control RNAi wherein GFP-CaMKIIα is at first relatively diffuse, but within 1 min, it translocates to the microtubules. This initial translocation is largely sustained for the remainder of the experiment.

By contrast, a neuron transfected with p600 RNAi (Fig. 2B, Suppl. movies 3 and 4) shows the same translocation of GFP-CaMKIIα to the microtubules, but this process is ostensibly less pronounced, and is interrupted by a sustained aggregation of GFP-CaMKIIα. The degree and time-course of aggregation of these representative neurons is quantified and plotted below. Figs 1 and 2 together show that there is a duration of depolarization after which the microtubule translocation of GFP-CaMKIIα is interrupted by self-aggregation, and that p600 sets the threshold at which the former behaviour transitions to the latter.

Stabilization of microtubules rescues the aggregation of GFP-CaMKIIα after direct depolarization

p600 directly binds and stabilizes microtubules [12]. We hypothesized that neurons in which p600 is depleted might have a less stable population of microtubules, making the translocation of GFP-CaMKIIα to these microtubules less probable. By replacing the microtubule-stabilizing function of p600 with 100 nM paclitaxel, we were able to significantly decrease the proportion of neurons showing GFP-CaMKIIα aggregation. At 5 s of depolarization, only 37.5% of neurons with p600 knockdown showed aggregation (6/16 with 100 nM paclitaxel, 37/37 with the vehicle control 0.01% DMSO; $p = 0.002$ according to the two-tailed Fisher's exact test). We conclude that during direct depolarization, p600 prevents GFP-CaMKIIα aggregation by stabilizing microtubules.

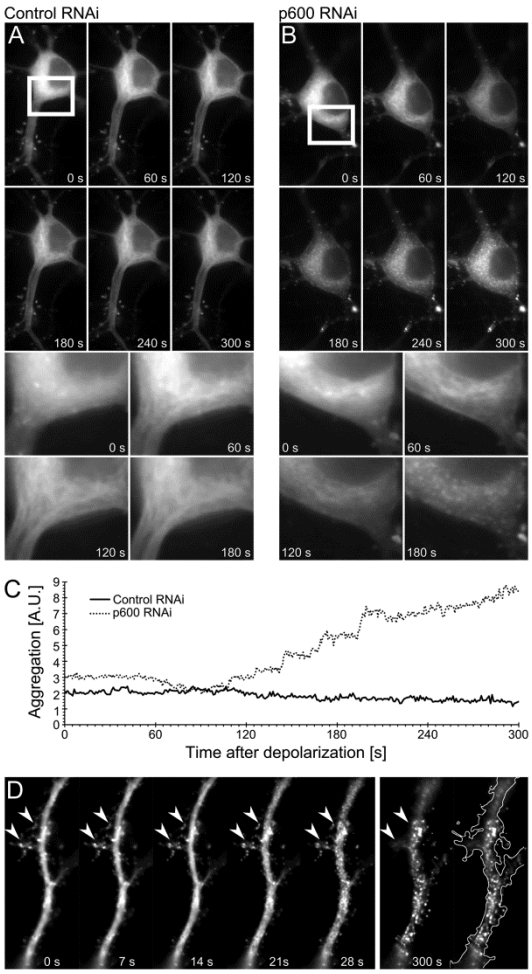

Fig. 2. In the absence of p600, the cytoskeletal localization of GFP-CaMKIIα is interrupted by a long-lasting self-aggregation. These are representative images. A and B – In a neuron transfected with control RNAi, upon depolarization, GFP-CaMKIIα undergoes a rapid and sustained translocation to the microtubules. When p600 is depleted, the cytoskeletal translocation of GFP-CaMKIIα is interrupted by a sustained aggregation. C – Frame-by-frame quantification of these movies shows that the aggregation of GFP-CaMKIIα takes place between 1 and 5 min after depolarization. D – Imaging of a single primary dendrite shows that the aggregation of GFP-CaMKIIα takes place primarily in the dendritic shaft.

GFP-CaMKIIα aggregation occurs primarily in the dendritic shaft

Lastly, we sought to compare the spatial dynamics of the CaMKIIα aggregation seen here to the synaptic translocation of CaMKIIα [18]. We used our live-imaging model to take high-resolution footage of the primary dendrite during depolarization. Fig. 2D and Suppl. movie 5 show that during depolarization, the aggregation of GFP-CaMKIIα takes place primarily in the dendritic shaft.

The overlay (on the final panel, in white) of the initial distribution of GFP-CaMKIIα over the final aggregated distribution illustrates that the aggregation involves a net (though incomplete) evacuation of dendritic protrusions. While we cannot conclude that the aggregation takes place exclusively outside of the synapses, we can reasonably claim that the aggregation of GFP-CaMKIIα seen in this study is not a synaptic phenomenon.

DISCUSSION

These data suggest a model wherein CaMKIIα translocates to microtubules during induced depolarization, and with a longer depolarization it begins to self-aggregate. In this model, p600 stabilizes microtubules in order to set the threshold at which CaMKIIα switches from microtubule translocation to self-aggregation. The notion that p600 regulates the localization of CaMKIIα by stabilizing microtubules is novel, and complements our previous study showing that p600 prevents CaMKII aggregation through binding to CaM in a microtubule-independent manner [17]. The effectiveness of microtubule stabilization for preventing CaMKIIα aggregation during direct depolarization, but not during glutamate treatment, led to a model wherein p600 has two modes of action depending on which channels are activated.

Based on the conservative assumption that the aggregation we have observed here is driven by Ca^{2+}, as it has been in every experimental paradigm so far, there are two possible mechanisms by which the microtubule-associated function of p600 could prevent CaMKIIα aggregation. The first general model is that the concentration of cytoplasmic Ca^{2+} is unaffected by the actions of p600, and that the response of CaMKII to the same stimulus is altered. Based on the interaction between p600 and CaMKIIα and the ubiquitous intracellular expression of both [17], it is possible that during voltage-dependent Ca^{2+} influx, CaMKIIα would normally target to p600 or to the stable microtubules it sustains. Depletion of p600 could remove these docking sites and thereby favour the self-aggregation of CaMKIIα.

The second possibility is that p600 regulates Ca^{2+} influx. Furukawa and Mattson [22] demonstrated that the stabilization of microtubules by paclitaxel can in fact restrict excitotoxic Ca^{2+} entry, and that microtubule destabilization causes neuronal death. Thus in addition to more well-known mechanisms by which cytosolic Ca^{2+} affects microtubule stability [24, 23], microtubule stability in turn affects the levels of cytosolic Ca^{2+}. By analogy to that study, p600 may stabilize microtubules during direct depolarization, restricting Ca^{2+} entry and preventing CaMKIIα aggregation. Though Furukawa and Mattson did not find that Ca^{2+} entry by KCl depolarization was mitigated by paclitaxel pre-treatment, they did find that the effect of microtubule stabilization on Ca^{2+} entry was channel-specific. This precedent of channel-specificity could offer means to reconcile the effectiveness of microtubule stabilization in direct depolarization that was not found after glutamate/glycine treatment.

The finding that p600 acts differently to prevent CaMKIIα aggregation when faced with different sources of Ca^{2+} strongly suggests a heterogeneous subcellular response by p600. Efforts to distinguish between these two general models will begin with detailed measurements of local cytosolic Ca^{2+} concentrations, and will require minute observations of microtubule dynamics when p600 is depleted.

It is noteworthy that the aggregation of CaMKIIα is distinct from its translocation to synapses [18]. In this report, the aggregation takes place both in the soma and the dendrites (Fig. 2B and D), as opposed to the purely synaptic targeting seen in the former case. Interestingly, the endogenous CaMKII inhibitor, CaMKII-N, resides exclusively in the dendritic shaft and soma [25]. Apart from the movement away from the synapses and into the dendritic shaft, there is a case for the inactivation of CaMKII. Firstly, the in vitro and in vivo inactivation of CaMKII follows a similar time-course with a completion time of about 5 min, which has been closely correlated to the aggregation and movement to the particulate fraction [4, 6, 26]. This time-course of inactivation and aggregation is strikingly similar to the aggregation quantified in Fig. 2C. Secondly, our previous work has shown that the depletion of p600 or disruption of the p600/CaM interaction not only causes CaMKIIα aggregation, but also causes neuronal death [17]. Since CaMKII tends to both inactivate and aggregate in excitotoxic conditions [4], the ultimately fatal outcome of p600 depletion suggests that the aggregation of CaMKIIα seen in this study also involves an inactivation.

Though the purpose of the CaMKIIα aggregates remains unknown, entry into this state can reasonably be supposed to preclude other physiological behaviours of CaMKIIα. Even allowing for the slight possibility that CaMKIIα in its aggregated form is able to interact with its usual binding partners, we can assume that these interactions would be greatly diminished on account of its sequestration in aggregates. For example, CaMKII directly phosphorylates the GluR1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor in order to potentiate synaptic strength [28, 27]. The implied inactivation of its kinase activity, the reduced mobility/larger size of the aggregates, and the net synaptic evacuation we observed (Fig. 2D) combine to make it unlikely that CaMKIIα aggregates are able to phosphorylate the AMPA receptor in any significant way. Similarly, aggregated CaMKIIα is unlikely able to maintain its usual role in binding and modulating the properties of NMDA receptors and L-type channels [29, 30]. The combined evidence strongly suggests that in its aggregated form, CaMKIIα is unable to continue in its role as a modulator of Ca^{2+}-signalling and synaptic plasticity. As a direct regulator of CaMKIIα aggregation, p600 is thereby a hypothetical regulator of these processes.

The consequence of interrupting the microtubule binding of CaMKIIα is more difficult to predict since it is not fully clear what function(s) the CaMKIIα/microtubule interaction serves. CaMKII, predominantly the α-isoform, associates with microtubule preparations, though it is not clear to what element

of microtubules it binds [31, 32]. However, it has been shown that CaMKIIα directly phosphorylates α-tubulin near the C-terminus [31, 32], thereby favouring the depolymerisation of microtubules [32]. This association and phosphorylation has even been proposed to be a means of directly encoding information on microtubules [33]. The only study of the functional consequences of the CaMKIIα/microtubule interaction [21] offered strong evidence that an interaction between CaMKIIα and dendritic microtubules increases the surface expression of AMPA receptors and spine remodelling. This study also showed that this translocation of CaMKIIα requires stable microtubules [21]. Although the intermediate mechanism is speculative, their result supports the model wherein activated CaMKIIα translocates to microtubules to ultimately potentiate synaptic strength. Therefore, p600 could be a regulator of synaptic strength by regulating the transition between the microtubules translocation and aggregation of CaMKIIα.

While CaMKIIα aggregation can be experimentally induced by all manner of pharmacological insults, the depletion of p600 or the mere disruption of the p600/calmodulin interaction [17] remains the only known means of inducing a neuron to enter this state by its own activity. p600 regulates the localization of CaMKIIα, but it is also required for neuronal survival [17].

While a general understanding of the role of CaMKIIα aggregation in neurons remains elusive, it is a topic that warrants further study both because of the importance of the enzyme itself, and as a proxy of neuronal death. This study also offers the first indication that the self-aggregation CaMKIIα is dependent on microtubule stability, and it introduces p600 as the mediator of this effect. Since Ca^{2+} overload, microtubule instability and p600 depletion have all been independently shown to cause neuronal death [17, 34, 35], this study puts us closer to a unified model wherein p600 is an interface between microtubule dynamics, Ca^{2+} signalling, and neuronal survival.

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