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Real-time imaging of NF-AT nucleocytoplasmic shuttling with a photoswitchable fluorescence protein in live cells

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ABSTRACT

Background: The transcription factor NF-AT plays a key role in the activation of many early immune response genes and is regulated by subcellular localization. NF-AT translocates from the cytoplasm to the nucleus then returns in response to the intracellular calcium level.
Methods: We have investigated NF-AT nucleocytoplasmic shuttling in real-time in living cells using NF-ATc1 tagged with the reversibly photoswitchable fluorescence protein, Dronpa. We monitored both nuclear import and export rate of Dronpa-tagged NF-AT in live cells upon stimulation with ionomycin plus calcium ($I+Ca^{2+}$) or cyclosporin A (CsA).
Results: The results show that NF-AT moved into the nucleus within 3–9 min after stimulation and moved back out into the cytoplasm within 15–50 min after CsA addition. In the absence of stimulation, NF-AT stayed in the cytoplasm as in the cells overexpressing GSK-3β, a calcineurin-opposing regulator.
General Significance: This semi-quantitative imaging with constant fluorescence provides the basis to detect the real-time effect by several regulators on NF-AT family proteins.

1. Introduction

In lymphoid cells, stimulation of Ca^{2+}–calcineurin signaling pathway leads to the nuclear translocation of the nuclear factor of activated T cells (NF-AT) family of transcription factors, which in turn activate immune response genes such as those encoding interleukin-2 (IL-2), IL-4, CD40 ligand, and Fas ligand [1]. NF-AT proteins contain an N-terminal regulatory domain that controls their nuclear import in response to calcineurin activation. In unstimulated cells, this regulatory domain is phosphorylated and masks the nuclear localization sequence, causing NF-AT proteins to be sequestered in the cytoplasm [2]. Calcineurin dephosphorylates the NF-AT regulatory domain, resulting a conformational change in the protein that promotes nuclear import [2]. Inhibition of the nuclear translocation of NF-AT is largely responsible for the immunosuppressive actions of cyclosporin A (CsA) and tacrolimus (FK506), which specifically inhibit calcineurin [3].

Regulation of NF-AT depends on the balance between inhibitory phosphorylation by nuclear export kinases such as glycogen synthase kinase (GSK)-3β, and stimulatory dephosphorylation by the calcium dependent serine/threonine phosphatase calcineurin [4,5]. GSK-3 is a ubiquitous serine/threonine protein kinase that phosphorylates a series of sites in the N-terminal regulatory regions of NF-AT proteins, promoting its export from the nucleus and antagonizing NF-AT dependent transcription [2].

The subcellular localization of NF-AT is an important component of their transcriptional activity. Previous study reported predominant nuclear localization of NF-ATc1 in COS cells upon stimulation with ionomycin plus calcium ($I+Ca^{2+}$) [6], which have not been quantitatively assessed in live cells. Although immunostaining can show protein locations in the steady-state, this might not represent the full picture and provides no information about protein dynamics. To overcome the limitation on investigating protein dynamics, we used the live cell fluorescence microscopy technique and a reversibly photoswitchable fluorescent protein, Dronpa as a genetically encodable tag to monitor. The Dronpa could be excited, erased, and excited again in a nondestructive manner, allowing direct observation of regulated shuttling of key signaling molecules without loss of fluorescence intensity [7,8]. By using this protein, the continuous monitoring of protein dynamics, such as nucleocytoplasmic shuttling of signaling proteins, becomes possible at multiple time points in individual cell.

In this study, we tagged NF-ATc1 with Dronpa (NF-ATc1-Dronpa) to measure the precise time course of NF-ATc1 nucleocytoplasmic shuttling directly and monitored its behavior in living cells upon stimulation with ionomycin plus calcium or cyclosporin A.

Our study provided the first real-time imaging of NF-ATc1 nucleocytoplasmic shuttling with high spatial and temporal resolution in living cells.

Abbreviations: NF-AT, nuclear factor of activated T cells; GSK-3, glycogen synthase kinase-3; CsA, cyclosporin A; $I+Ca^{2+}$, ionomycin plus calcium

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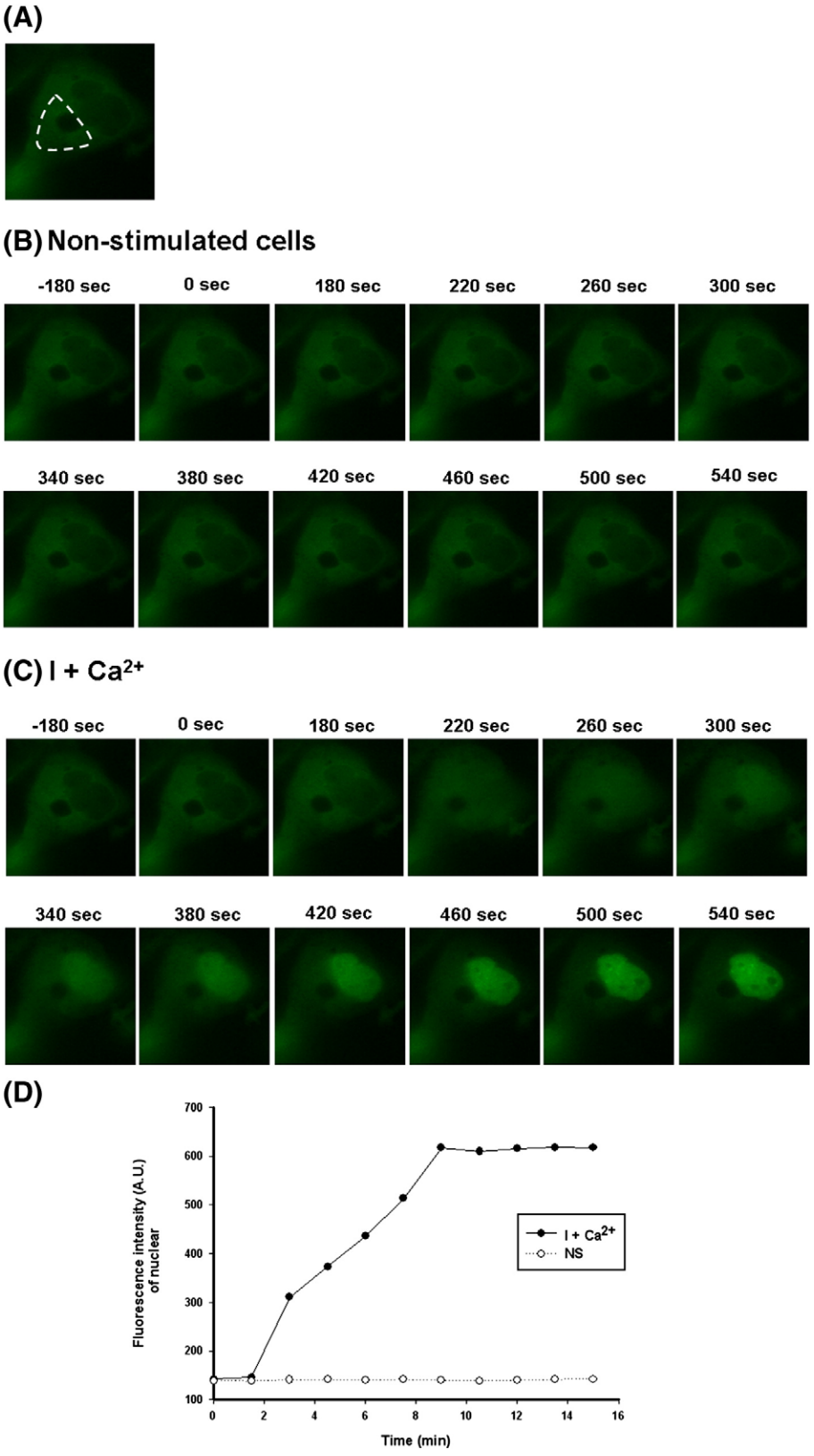

Fig. 1. Monitoring the nuclear import of NF-ATc1-Dronpa in COS7 cells. Cells were transiently transfected with NF-ATc1-Dronpa, treated with 2 μM ionomycin plus 10 mM calcium ($I+Ca^{2+}$) for the indicated time. (A) The region photoactivated with a 436±10 nm filter is shown in dotted line. The Dronpa was photoactivated in part of the cytoplasm for monitoring nuclear import. (B, C) Nuclear import of NF-ATc1-Dronpa in non-stimulated cells (B) or after 180 s stimulation with $I+Ca^{2+}$ (C). (D) Time courses of the nuclear import rates of NF-ATc1-Dronpa. The nuclear import rates of NF-ATc1-Dronpa were measured at all time points by analyzing the intensities of the average Dronpa fluorescence signal in the nucleus, obtained from three different cells.

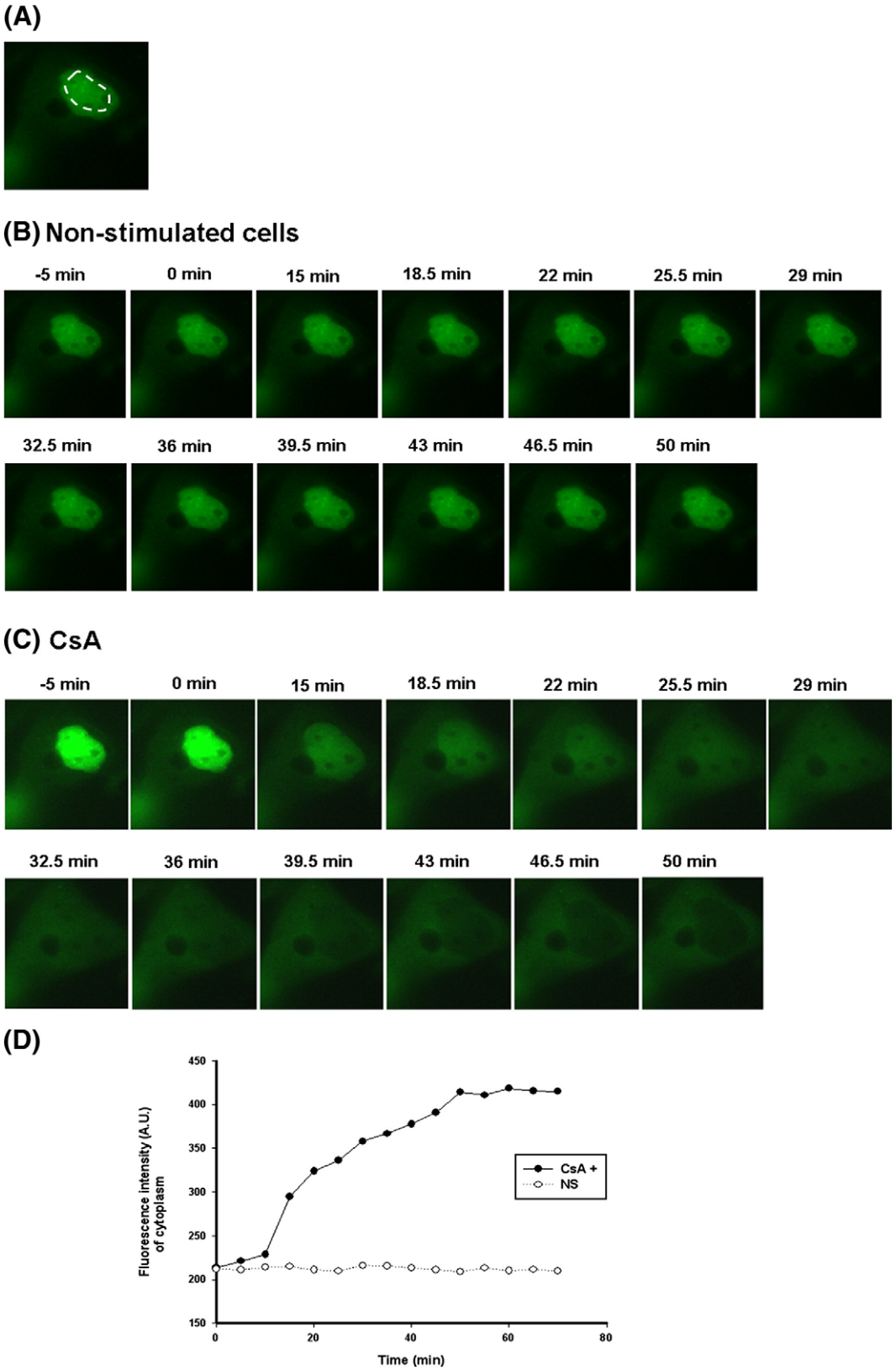

Fig. 2. Monitoring the nuclear export of NF-ATc1-Dronpa in COS7 cells. Ca^{2+} stimulation was terminated by replacing medium with the medium containing 2 μg/ml Cyclosporin A (CsA) to export. (A) The region photoactivated with a 436±10 nm filter is shown in dotted line. The Dronpa was photoactivated in the nucleus for monitoring nuclear export. (B, C) Nuclear export of NF-ATc1-Dronpa in non-stimulated cells (B) or after 15 min stimulation with CsA (C). (D) Time courses of the nuclear export rates of NF-ATc1-Dronpa. The nuclear export rates of NF-ATc1-Dronpa were measured at all time points by analyzing the intensities of the average Dronpa fluorescence signal in the cytoplasm, obtained from three different cells.

2. Materials and methods

2.1. Plasmid construction

The pcDNA3NF-ATc1 plasmid was kindly provided by Dr. Hiroshi Takayanagi (Tokyo Medical and Dental University). The full-length NF-ATc1 was amplified from a pcDNA3NF-ATc1 plasmid with forward (5′-CCGGAATTCGGCCACCATGCCAAATACCAGCTTT-3′) and reverse (5′-CCCAAGCTTGTAAAAACCTCCTCTCAG-3′) as primers. The PCR product was cloned into the EcoRI/HindIII site of pDG1-MN1 encoding the CoralHue™ Dronpa-Green1 (DG1) fluorescent protein (MBL, Japan). The DNA constructs were confirmed by DNA sequencing.

2.2. Cell culture, transfection and stimulation

COS7 cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, 100 μg/ml of streptomycin at 37 °C in 5% CO_2. Transient transfection of the cells was performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were passaged 24 h prior to transfection and imaged 48 h post-transfection. For NF-ATc1 translocation studies, cells were stimulated with 2 μM ionomycin (Calbiochem) and 10 mM $CaCl_2$ (Sigma) to promote import. Ca^{2+} stimulation was terminated by replacing the medium with medium containing 2 μg/ml Cyclosporin A (CsA, Sigma) to export.

2.3. Fluorescence microscopy and live cell imaging

Microscopy was preformed on a DeltaVision system manufactured by Applied Precision (Issaquah, WA, USA). The sample was imaged using a microscope (IL-70, Olympus) equipped with a 100 W mercury arc lamp, a Uplan Apo 60× oil objective (1.35 numerical aperture), a CoolSnap HQ digital camera from Roper Scientific (Tucson, AZ, USA) and optical filter sets from Omega Optical (Brattleboro, VT, USA). For live cell imaging, the prepared cells were placed in a temperature-controlled chamber equipped on the fluorescence microscope. Cells were maintained at 37 °C and humidified 5% CO_2. Intermediately bright cells were chosen for imaging experiments. COS-7 cells expressing NF-ATc1-Dronpa were imaged on a microscope. Exposure times were 200 ms with 2×2 binning and a final image size of 512×512. The system operates a 436±10 nm and a 490±20 nm filters independently, enabling the simultaneous execution of photoactivation or photobleaching together with fluorescence observation. The fluorescence of Dronpa was erased through a 30% transmittance neutral density (ND) filter at 490±20 nm for 20 s, followed by its photoactivation by excitation at 436±10 nm for 200 ms. Each image was taken at every 40 s for 20 min (for nuclear import) or 3.5 min for 70 min (for nuclear export) after activation using the same filter. Images were analyzed with the SoftWoRx program from Applied Precision.

3. Results and discussion

3.1. Real-time imaging for nuclear import and export of NF-ATc1-Dronpa in live cells

NF-AT nucleocytoplasmic shuttling is a key event in calcineurin signaling and in exerting transcriptional activity on the regulation of early immune response genes. Previous studies with immunocytochemistry or GFP-fused NF-AT have examined nuclear accumulation upon Ca^{2+} ionophore stimulation or the steady-state distribution of NF-AT between the nucleus and cytoplasm [1,6,9]. However, these conventional methods do not provide enough information about spatial and temporal dynamics of NF-AT based on Ca^{2+} signaling in living cells. Compared with fixed cell labeling methods that provide snapshots of activity, such as immunocytochemistry, live cell imaging enables to obtain real-time visualization. In order to directly measure the exact nuclear import and export time of NF-ATc1, we quantified

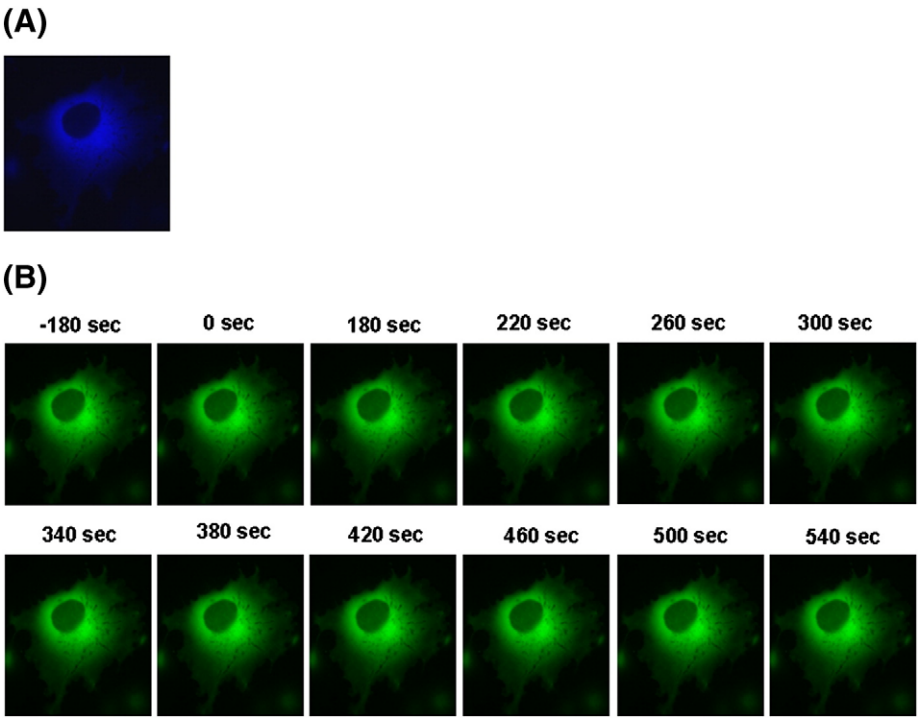

Fig. 3. Inhibition of nuclear localization of NF-ATc1 by overexpression of GSK-3β. NF-ATc1-Dronpa was coexpressed with GSK-3β in COS7 cells. Cells were treated with 2 μM ionomycin and 10 mM calcium to induce nuclear localization of NF-ATc1 as done for the Fig. 1. (A) Subcellular localization of GSK-3β-CFP in COS7 cells. (B) The time periods monitored for nuclear import of NF-ATc1-Dronpa during stimulation with I+Ca^{2+} in COS7 cells cotransfected with NF-ATc1-Dronpa and GSK-3β. Results represent a typical experiment from three independent trials.

the shuttling rate of NF-ATc1 in real-time using NF-ATc1 tagged with the reversibly photoswitchable fluorescence protein, Dronpa (NF-ATc1-Dronpa). Dronpa is monomeric, displays favorable switching properties, and shows bright fluorescence with a remarkable fluorescence quantum yield [7]. Dronpa is photobleached by excitation at 490 nm, but its fluorescence can be restored with 400 nm irradiation [7,8]. In contrast to conventional GFP variants, Dronpa is rapidly photobleached with minimal light intensity (and thus much less photodamage and nonspecific effects) and can be reactivated and rebleached repeatedly [7,10]. The perfect photochromic characteristics of Dronpa provide an unprecedented molecular tool for studying fast protein dynamics and a multitude of biochemical signals in living cells and organisms. Dronpa has been successfully used for several protein tracking studies for this reason [7,11,12]. Employing the photochromic properties of Dronpa, we monitored both nuclear import and export of Dronpa-tagged NF-AT in live COS7 cells upon stimulation with ionomycin plus calcium or cyclosporin A.

NF-ATc1-Dronpa was initially distributed throughout the cytoplasm of COS7 cells which were chosen for easier assessment of cytoplasmic and nuclear localization. For nuclear import studies of NF-ATc1-Dronpa, cells were stimulated with ionomycin and calcium for the indicated time. After fluorescence was erased to background levels with a 490±20 nm filters, Dronpa was photoactivated in the cytoplasm using a 436±10 nm filters, then a series of images was acquired by using a 490±20 nm filters. The nuclear import was examined by tracing the fluorescence of NF-ATc1-Dronpa in the cytoplasm to the nucleus. At early time points, the nuclear import remained slow; however, 3 min after the onset of Ca^{2+} ionophore stimulation, nuclear import was greatly enhanced. Transfected NF-ATc1-Dronpa moved into the nucleus within 3–9 min after Ca^{2+} ionophore treatment (Fig. 1C). The flow rate of nuclear import was quantified and plotted against time (Fig. 1D). In the absence of stimulation, NF-ATc1-Dronpa was located in the cytoplasm (Fig. 1B).

The drugs cyclosporin A (CsA) and FK506 exert their immunosuppressive effects by inhibiting calcineurin [4], leading to block the nuclear translocation of NF-ATc1 [13].

To examine the nuclear export rate of NF-ATc1-Dronpa, we terminated Ca^{2+} signaling by changing the medium with cyclosporin A containing medium and acquired images. After erasure, nuclear export was examined by tracing the fluorescence of NF-ATc1-Dronpa photoactivated in the nucleus to the cytoplasm. In real-time imaging, nuclear export was accelerated 15 min after the termination of Ca^{2+} signaling (Fig. 2C). NF-ATc1-Dronpa moved back out to the cytoplasm within 15–50 min after the termination of Ca^{2+} signaling observed by live cell imaging (Figs. 2C and D). In non-stimulated cells (NS), NF-ATc1-Dronpa was located in the nucleus (Fig. 2B). These results confirmed that the nuclear import rate of NF-ATc1 is more rapid than its export. More importantly, we were able to determine the exact rate of nuclear import and export of NF-ATc1 by quantification of time and fluorescence for the first time.

Using live cell imaging and Dronpa, we visualized under a fluorescence microscope when, where, and how the NF-ATc1 is moved in single living cells. Based on the quantitative data from live cell imaging, a dynamic alteration in subcellular localization of signaling molecules can be revealed in response to various extracellular and intracellular environments. Our method would be a useful tool to analyze the heretofore unexplored regulation of fast protein dynamics and permit precise time course of nucleocytoplasmic shuttling of signaling proteins.

3.2. Inhibition of nuclear localization of NF-ATc1 by overexpression of GSK-3β

In nonexcitable cells, bidirectional shuttling of NF-ATc proteins between cytoplasm and nucleus is regulated by calcineurin and opposing kinases. NF-AT belongs to a family of highly phosphorylated transcription factors. Several Ser/Thr kinases, including GSK-3β, JNK, MEKK and casein kinase 1α phosphorylate NF-AT proteins at specific amino terminal motifs, thereby opposing the actions of calcineurin [14].

We examined the subcellular distribution of NF-ATc1 using real-time imaging method to determine how in time course activated GSK-3β prevented nuclear accumulation of NF-AT in the presence of activated calcineurin. Glycogen synthase kinase-3 (GSK-3) is a highly conserved proline directed serine/threonine kinase that phosphorylates NF-AT in vivo and opposes Ca^{2+}/calcineurin induced nuclear entry [1]. NF-ATc1-Dronpa and GSK-3β were transiently expressed in COS7 cells and the cells were treated with ionomycin and calcium to localize NF-ATc1-Dronpa to the nucleus. GSK-3β was detected throughout the cytoplasm and was almost completely co-localized with NF-ATc1-Dronpa in the transiently transfected COS7 cells (Fig. 3A). When NF-ATc1-Dronpa and GSK-3β were coexpressed in COS7 cells, NF-ATc1-Dronpa was localized in cytoplasm and did not move at all within 3–9 min after the stimulation (Fig. 3B). It was obvious that overexpression of GSK-3β blocked the Ca^{2+}–calcineurin induced nuclear translocation of coexpressed NF-ATc1-Dronpa in COS7 cells. This result implies that GSK-3β acts on controlling the nuclear export of NF-ATc1 and that our method will be able to get utilized to fast-screen for agents and proteins that selectively inhibit this process.

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