Kinetics of nuclear-cytoplasmic translocation of Foxo1 and Foxo3A in adult skeletal muscle fibers

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Schachter TN, Shen T, Liu Y, Schneider MF. Kinetics of nuclear-cytoplasmic translocation of Foxo1 and Foxo3A in adult skeletal muscle fibers. Am J Physiol Cell Physiol 303: C977–C990, 2012. First published August 29, 2012; doi:10.1152/ajpcell.00027.2012.—In skeletal muscle, the transcription factors Foxo1 and Foxo3A control expression of proteins that mediate muscle atrophy, making the nuclear concentration and nuclear-cytoplasmic movements of Foxo1 and Foxo3A of therapeutic interest in conditions of muscle wasting. Here, we use Foxo-GFP fusion proteins adenovirally expressed in cultured adult mouse skeletal muscle fibers to characterize the time course of nuclear efflux of Foxo1-GFP in response to activation of the insulin-like growth factor-1 (IGF-1)/phosphatidylinositol-3-kinase (PI3K)/Akt pathway to determine the time course of nuclear influx of Foxo1-GFP during inhibition of this pathway and to show that Akt mediates the efflux of nuclear Foxo1-GFP induced by IGF-1. Localization of endogenous Foxo1 in muscle fibers, as determined via immunocytochemistry, is consistent with that of Foxo1-GFP. Inhibition of the nuclear export carrier chromosome region maintenance 1 by leptomycin B (LMB) traps Foxo1 in the nucleus and results in a relatively rapid rate of Foxo1 nuclear accumulation, consistent with a high rate of nuclear-cytoplasmic shuttling of Foxo1 under control conditions before LMB application, with near balance of unidirectional influx and efflux. Expressed Foxo3A-GFP shuttles ~20-fold more slowly than Foxo1-GFP. Our approach allows quantitative kinetic characterization of Foxo1 and Foxo3A nuclear-cytoplasmic movements in living muscle fibers under various experimental conditions.

nuclear influx; leptomycin B; PP2A; IGF-1; Akt
The responses of endogenous Foxo1 and adenovirally expressed Foxo1-GFP to the treatments described above identifies our model system as being an accurate and useful tool in the kinetic study of changes in subcellular distribution of Foxo1 in skeletal muscle fibers.

**MATERIALS AND METHODS**

**Materials.** OA, staurosporine, and IGF-1 were purchased from Sigma Aldrich (St. Louis, MO) and leptomycin B from LC Laboratories (Woburn, MA). The Akt inhibitor Akt-I-1,2 and the PI3K

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**Fig. 1.** Subsarcomeric distribution of endogenous Foxo1 and exogenous Foxo1-GFP. Representative confocal images of immunocytochemistry assays of endogenous Foxo1 (A) and fluorescence of Foxo1-GFP (B) with α-actinin establish its Z-line localization. A and B, top: large section of a fiber with a red square indicating the segment of the fiber that is magnified at bottom. Below each enlarged region is a graph demonstrating the total fluorescence of the enlarged region as a function of distance as detailed on the x-axis. Scale bars = 10 μm.
inhibitor LY294002 were obtained from Calbiochem (Darmstadt, Germany).

Isolation and culture of adult flexor digitorum brevis muscle fibers. The flexor digitorum brevis (FDB) was isolated from adult female CD1 mice (4–6 wk old). Animals were euthanized by asphyxiation via CO₂ followed by cervical dislocation according to protocols approved by the University of Maryland Institutional Animal Care and Use Committee. Individual fibers were enzymatically dissociated and cultured using a modified protocol previously described in Liu et al. (18). Briefly, the muscle was incubated in MEM (Invitrogen, Carlsbad, CA) containing 3.5 μg/ml collagenase type I (Sigma-Aldrich), 10% FBS, and 50 μg/ml gentamicin for 2 h at 37°C to enzymatically dissociate the muscle. Manual manipulation to separate individual fibers was done by triturating the muscle gently in media containing no collagenase. Approximately 50 fibers were then plated in a laminin-coated glass-bottomed dish.

Adenoviral infection of cultured FDB fibers. Fibers were plated in 2 ml serum-free MEM with 9.2 × 10⁸ plaque-forming units/μl adFoxo1-GFP or 4.8 × 10⁸ plaque-forming units/μl adFoxo3A-GFP lysate and incubated for 48–72 h. The adenovirus encoding Foxo1-GFP was a gift from Dr. Joseph Hill (University of Texas Southwestern Medical Center; Ref. 20), and the Foxo3A-GFP adenovirus was purchased from Applied Biological Materials (Richmond, British Columbia, Canada; catalog no. 000420A). Both adenoviruses were amplified in our laboratory. In both constructs, the GFP fusion tag is attached at the COOH-terminal of Foxo.

Confocal fluorescence imaging of living cultured adult muscle fibers. A half hour before imaging, the culture dish was removed from the incubator and the culture media were removed and replaced with L-15 (Invitrogen). The culture dish was then set on the stage of an Olympus IX70 inverted microscope equipped with an Olympus FLUOVIEW 500 laser scanning confocal imaging system with excitation wavelengths of 488 and 647 nm. Fibers were viewed with an Olympus ×60/1.2 NA water-immersion objective and scanned at zoom 1) using consistent laser output and gain. Then, 30 Aş, Olympus FLUOVIEW 500 laser scanning confocal imaging system with excitation wavelengths of 488 and 647 nm. Fibers were viewed with an Olympus ×60/1.2 NA water-immersion objective and scanned at zoom 1) using consistent laser output and gain. Then, 30 min after media change to L-15, images were taken for 30 min at 10-min increments to establish a baseline in each individual fiber. After the last baseline image was taken, LMB or other treatment was added to the dish and time was set to begin from 0 min at that point. Once the experiment began, the medium was not removed at any point; only additions of small volumes of reagents were made.

In cases of strong nuclear uptake of Foxo1-GFP, including the fiber in RESULTS (see Fig. 3B), the nuclei of the fiber reached saturation of our detection system using the control laser intensity. Therefore, continued imaging used decreased laser intensity to avoid nuclear saturation. However, the images shown in RESULTS (see Fig. 3) were taken at the original laser intensity with saturated nuclei for qualitative comparison to show the overall extent of the effect. The change in laser intensity when saturation occurs does not affect our quantification of nuclear concentration because we calculate the nuclear concentration as a nuclear to cytoplasmic fluorescence ratio (n/c), and both the nuclear fluorescence and cytoplasmic fluorescence are affected by the same percent by change in laser intensity.

Image analysis. The mean pixel fluorescence of the cytoplasm and nucleus from each image was quantified using an area of interest in Image J as indicated in RESULTS (see Fig. 3, A and C), and then the background mean pixel fluorescence was subtracted from each. The ratio of nuclear mean pixel fluorescence to cytoplasmic mean pixel fluorescence (n/c ratio) is calculated for each time point to allow comparison of nuclear fluorescence independent of expression levels of Foxo1-GFP and should be proportionate to nuclear concentration normalized to cytoplasmic level of expression. Student’s t-tests were used for comparisons of data obtained from two experimental conditions, and differences were considered significant if P < 0.05.

Fluorescence immunocytochemistry. Fluorescence immunocytochemistry was carried out as in Shen et al. (28). Briefly, fibers were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X-100 in PBS, and then blocked with 5% normal goat serum. Fibers were incubated with anti-Foxo1 (no. 2880; Cell Signaling), anti-α-actinin (Sigma), or anti-nucleophosmin (Zymed, San Francisco, CA) overnight followed by overnight incubation with a fluorescent-tagged secondary antibody. The stained fibers were imaged using the confocal microscope and lasers described above. Colocalization of immunofluorescence images were merged, and mean pixel fluorescence were measured as a function of distance for tracings and enhanced using ImageJ. No other image in this study was enhanced.

Western blotting. Protein extraction and Western blotting techniques were performed as described in Shen et al. (31). Briefly, FDB were cultured for 2 days and then treated for 80 min as indicated. Fibers were then collected and mixed with M-PER (Thermo Scientific, Rockford, IL) and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and passed through a 21-gauge syringe several times, followed by high speed centrifugation. The supernatant was combined with sample reducing agent and LDS sample buffer (Invitrogen), heated at 70°C for 10 min, and run on a NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen). Proteins were transferred to a PVDF membrane. Akt antibody (no. 9272; Cell Signaling) and phosphospecific Akt antibody (no. 9271; Cell Signaling) were used and the membrane was then treated with ECL and film was exposed and developed.

RESULTS

Adenovirally expressed Foxo1-GFP is distributed in a manner consistent with endogenous Foxo1 in adult muscle fibers. To establish a live adult muscle fiber system to explore the...
phosphorylation dependency of the kinetics of Foxo1 nuclear-cytoplasmic translocation in skeletal muscle, we infected cultured adult FDB fibers with an adenovirus coding for Foxo1-GFP, which can be tracked quantitatively in subcellular regions of living muscle fibers using fluorescence confocal microscopy. To validate this system, we first compared the sarcomeric localization as well as nuclear/cytoplasmic distribution of endogenous Foxo1 to that of adenovirally expressed Foxo1-GFP (Fig. 1). Using immunocytochemistry, we established the sub-sarcomeric colocalization of endogenous Foxo1 with α-actinin (Fig. 1A, right), a well-established Z-line protein. Foxo1-GFP also colocalized with α-actinin (Fig. 1B, right), demonstrating consistent Z-line localization of both expressed Foxo1-GFP and endogenous Foxo1. In agreement with these findings, antibody staining of Foxo1 and the fluorescence of Foxo1-GFP in fibers expressing Foxo1-GFP displayed colocalization (data not shown). Under resting conditions, Foxo1-GFP is also present in the nuclei in a generally diffuse pattern (Fig. 1B, left) but does not enter the nucleolus (data not shown).

We next compared the n/c ratios of immunostained Foxo1-GFP and endogenous Foxo1 under control conditions. The normalization to cytoplasmic levels provides a means of comparing the concentrations of nuclear Foxo1 in a manner that is not expression dependent. The n/c ratios attained using immunocytochemistry of endogenous Foxo1 agreed very closely with n/c ratios of immunostained Foxo1-GFP under control conditions (Fig. 2A).

To further characterize our conditions, we compared the cytoplasmic anti-Foxo1 fluorescence levels in fibers expressing Foxo1-GFP and in noninfected control fibers. We treated both sets of fibers with anti-Foxo1 primary antibody and conjugated Alexa-647 secondary antibody (which does not interfere with GFP emissions) and found that the total Foxo1 cytoplasmic concentration in infected fibers was approximately sevenfold that of uninfected fibers (Fig. 2B). A typical area of interest used for determining the mean pixel fluorescence of the cytoplasm in a confocal image for a given fiber is shown in white at left in Fig. 3A and that for a nucleus is shown in white in Fig. 3C.

The similarity in sarcomeric localization and nuclear/cytoplasmic distribution of expressed Foxo1-GFP and endogenous Foxo1, coupled with consistency in response to phosphorylating and dephosphorylating agents (see Fig. 4, bottom) leads us to conclude that adenovirally expressed Foxo1-GFP is a good model for endogenous Foxo1. The system of adenovirally expressed Foxo1-GFP in cultured adult skeletal muscle fibers is thus a useful tool for real-time monitoring of kinetics of translocation of Foxo1 in live cells in a quantitative manner.

Nuclear-cytoplasmic movements of Foxo1 are kinase dependent. Under the standard conditions used for these studies, fibers exposed to adenovirus Foxo1-GFP were cultured in serum-free media without added growth factors for 48–72 h. The media were then changed to L-15 imaging media (as described in MATERIALS AND METHODS) with no added growth factors or serum. Treatment with 100 ng/ml IGF-1, a concentration that produces both myogenic and myogenic responses in cultured myoblasts (11), caused a rapid and marked reduction in the concentration of nuclear Foxo1-GFP (Fig. 3, A and C). After 20 min of IGF-1 treatment, nuclear/cytoplasmic Foxo1-GFP decreased to 20% of control and by 40 min reached a steady level of 10% of control (Fig. 4A, squares). In comparison, a gradual increase in nuclear/cytoplasmic fluorescence of Foxo1-GFP was observed over the same time period in control fibers where no changes were made to the medium bathing the fibers

![Fig. 3. Broad spectrum kinase inhibitor staurosporine promotes Foxo1 nuclear entry. Representative confocal images of single fibers at 0 and 80 min of insulin-like growth factor-1 (IGF-1; A) or staurosporine (B) treatments, as labeled. White arrows point to nuclei. Over 80 min of IGF-1 treatment the nuclear concentration of Foxo1 nuclei decreases visibly whereas the nuclei of the fiber treated with staurosporine increase to the point of saturation. White box indicates an average cytoplasmic region used to quantify cytoplasmic fluorescence. Scale bars are 20 μm. Magnification of individual nuclei from control fibers, a fiber treated with IGF-1 (C), and a fiber treated with staurosporine (D), as labeled. Fluorescence of the control nuclei increase very slightly whereas IGF-1 treatment causes a decrease in nuclear fluorescence and staurosporine treatment causes an increase in nuclear fluorescence. White outline in C indicates the nuclear region used to quantify nuclear fluorescence. Scale bars = 5 μm.](http://ajpcell.physiology.org/)
This slow increase in nuclear/cytoplasmic Foxo1-GFP fluorescence is likely due to the previous removal of the culture media, which presumably contained secreted autocrine/paracrine growth factors produced by fibers during the 48–72 h of fiber culture (22), and the subsequent addition of growth factor-free imaging media. The cytoplasmic fluorescence showed little change with time when IGF-1 was included in the imaging media (data not shown).

Treatment with 1 μM staurosporine, a nonselective kinase inhibitor, had the opposite effect from IGF-1; it caused a rapid increase in nuclear Foxo1 (Figs. 3, B and D, and 4B). After 20 min, the nuclear concentration of Foxo1 increased by 40%, and over 80 min it increased by 144% (Fig. 4B). The opposite changes in nuclear Foxo1-GFP in response to treatment with IGF-1, an upstream activator of several kinases including Akt, and staurosporine, a nonselective kinase inhibitor, demonstrate the phosphorylation dependence of nuclear fluxes of Foxo1. PI3K/Akt pathway is necessary for Foxo1 phosphorylation. To determine the pathway(s) involved in the phosphorylation of Foxo1, which regulates the nuclear-cytoplasmic fluxes of Foxo1.
Foxo1, we employed specific kinase inhibitors. 25 μM LY294002, a specific PI3K inhibitor, induced an increase in nuclear Foxo1-GFP within 40 min (Fig. 4C). Akt-I-1,2 is a selective inhibitor of Akt 1 and Akt 2 that does not cause significant inhibition of other kinases with the exception of CaMK1, a kinase that, to our knowledge, does not affect the phosphorylation status of Foxo1 (2, 3). The Akt inhibitor, Akt-I-1,2 (1 μM; Fig. 4D), also caused an increase in nuclear Foxo1-GFP. As determined by western blot and a phospho-specific Akt antibody, 80 min of treatment with staurosporine, LY294002, or Akt-I-1,2 each efficiently inhibited phosphorylation of Akt (Fig. 4E). These data indicate that the PI3K/Akt pathway is necessary to block Foxo1 nuclear entry.

We also compared the relative values of n/c ratios obtained for Foxo1-GFP and for endogenous Foxo1 under control conditions to n/c ratios in the presence of phosphorylating agents and phosphorylation inhibitors and determined that the ratios are similar under the same condition, further validating the use of exogenous Foxo1-GFP to monitor Foxo1 movement in living fibers (Fig. 4, F–G).

Inhibition of PP2A via OA decreases nuclear Foxo1. The phosphatase PP2A has been shown to directly dephosphorylate Foxo1 in the FL5.12 cell line expressing doxycycline-inducible wild-type Foxo1 (37). Treatment of cultured muscle fibers with 100 nM OA, a selective inhibitor of the PP2A class of phosphatases at this concentration (33), drastically reduced nuclear Foxo1 and inhibited the increase in nuclear Foxo1 that occurs with time in control fibers (Fig. 5, A and B), implicating PP2A as a Foxo1 phosphatase in skeletal muscle.

Nuclear-cytoplasmic shuttling of Foxo1. The results presented thus far show changes in net nuclear Foxo1 resulting from differences between nuclear influx and efflux of Foxo1. However, the magnitudes of the simultaneously occurring nuclear efflux and influx underlying the observed net flux was not determined. Treatment with a maximally effective concentration of LMB, an irreversible inhibitor of the export carrier CRM1, should eliminate nuclear export of Foxo1. Under this condition, the time course of the resulting buildup of nuclear Foxo1 would then occur at a rate equal to its rate of unidirectional flux out of the cytoplasm and into the nucleus. Therefore, using LMB we can calculate the rate of nuclear influx of Foxo1 under various conditions. Under resting conditions, nuclear influx of Foxo1-GFP occurs at a fast pace in LMB. During 80 min of exposure to 40 nM LMB, nuclear Foxo1 increased 10-fold (Fig. 6, A and B). Because there is no substantial buildup of Foxo1 without LMB under the same (control) conditions, we conclude that fast shuttling of Foxo1 into and out of the nucleus occurs in the absence of LMB; 20, 40, and 80 nM LMB induced the same rate of nuclear buildup of Foxo1, indicating that CRM-1 is maximally inhibited by 40 nM LMB under these conditions (Fig. 6C).

In contrast to our studies without LMB, where Foxo1-GFP is not strongly accumulated in the muscle fiber nuclei, during LMB treatment Foxo1-GFP can become highly concentrated in the nuclei and the cytoplasmic Foxo1-GFP fluorescence visibly decreases. Over 80 min of LMB treatment, the cytoplasmic fluorescence is reduced by about half (Fig. 6D), whereas in control conditions without LMB treatment, the cytoplasmic fluorescence does not change noticeably (data not shown).

By conservation of mass,

\[
\Delta c V_c = -\Delta n V_n, \tag{1}
\]

where \(\Delta c\) and \(\Delta n\) are the corresponding changes in c and n when a given amount of Foxo1-GFP moves between the cytoplasm and the nuclei of a muscle fiber and \(V_c\) and \(V_n\) are the cytoplasmic and nuclear volumes. The mean value of \(-\Delta c/\Delta n\) obtained from 11 fibers exposed to LMB for 80 min was 0.05 ± 0.001, which equals the mean value of \(V_c/V_n\) in these muscle fibers.

Role of cytoplasmic phosphatase. The PP2A inhibitor OA induces a decrease in nuclear Foxo1 in the absence of LMB (Fig. 5, A and B). To determine the manner in which this nuclear decrease occurs, fibers were or were not pretreated with OA for 30 min, followed by the addition of LMB. Using LMB to determine the rate of nuclear import of Foxo1, we established that OA effectively inhibits nuclear influx of Foxo1 (Fig. 6E). During 60 min of OA and LMB treatment, the nuclear concentration of Foxo1 increased linearly with a slope

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Fig. 5. PP2A inhibitor okadaic acid (OA) reduces Foxo1-GFP nuclear influx. A: representative confocal images of 2 fibers at 0 and 80 min of 100 nM okadaic acid treatment (bottom) or under control conditions (top). Scale bars = 5 μm. B: quantification of n/c ratio of Foxo1-GFP vs. time during 100 nM OA (n = 5) or control (n = 4) treatment demonstrates OA ability to decrease nuclear Foxo1. Data represent means ± SE.
of 0.04 n/c per min compared with control fibers treated with only LMB, where the slope was 0.19 n/c per min (Fig. 6E). After 60 min of OA and LMB treatment, nuclear Foxo1 became constant. In contrast, in fibers treated with LMB alone, n/c ratio continued to increase linearly during 60 min of LMB exposure, and showed a >10-fold increase over that of the nuclear concentration at 0 min, the point of addition of LMB (Fig. 6E). Based on these data, we conclude that Foxo1 nuclear import is induced by cytoplasmic dephosphorylation of Foxo1 via an OA-sensitive phosphatase, presumably PP2A.

Near balance between relatively large nuclear influx and nuclear efflux under resting conditions. In previous studies from our own and other laboratories (21, 29), the observation of a rapid net nuclear influx during application of the CRM1-dependent nuclear efflux blocker LMB has been taken as evidence for the presence of relatively large, but near balanced, nuclear influx and efflux before the addition of LMB. However, we are unaware of any previous reports of direct comparison of unidirectional influx and efflux rates under conditions of such nuclear-cytoplasmic shuttling of any molecule in any cell type. We therefore carried out experiments to directly address this point using our muscle fiber culture system. To improve time resolution, in these studies we used more frequent image acquisition, now at 2-min intervals, compared with the preceding results that were based on images acquired at 10 or 20 min intervals. We first monitored fibers under control conditions and then added either a maximally blocking concentration of LMB (Fig. 7, grey line) or a highly effective concentration of IGF-1 (Fig. 7, black line). The increase in nuclear Foxo1-GFP due to LMB addition begins with little delay, as expected for a direct, diffusion-limited pharmacological block of the nuclear export system by LMB. Subsequently, the nuclear accumulation of Foxo1 continues at a constant rate for the 40-min recording interval after LMB addition. In contrast, the decrease in nuclear Foxo1-GFP on addition of IGF-1 begins with a clear time lag. This is as expected for the occurrence of a multistep signaling cascade initiated by IGF-1 but accomplished by the sequential activation of IGF receptor, PI3K, and Akt, leading to the eventual phosphorylation of Foxo1, which eliminates Foxo1 nuclear influx and promotes Foxo1 net nuclear efflux.

To obtain a rough measure of the unidirectional flux rates under control conditions in Fig. 7, we used the mean flux rate from 10 to 20 min after reagent addition. This gives a unidirectional influx rate of 0.16 ± 0.03 n/c per min (means ± SE) in 9 nuclei from 8 fibers from 2 experiments as in Fig. 7 in the
We also noted that the sarcomeric pattern of distribution of Foxo3A was different from that of Foxo1. Whereas Foxo1-GFP was localized in a single sharp line per sarcomere at the sarcomeric Z-lines (Fig. 1B), Foxo3A-GFP is present in a doublet band per sarcomere on either side of the Z-line (Fig. 8D).

Rate of unidirectional nuclear influx. To determine the effect of kinase activity on the unidirectional rate constants for movement of cytoplasmic Foxo1-GFP out of the cytoplasm and into the nucleus, after a control period of 30 min we pretreated fibers with LMB for 40 min and then added staurosporine or IGF-1 for an additional 120 min (Fig. 9, A and B). The average rate of increase of nuclear fluorescence in LMB alone was 0.14 n/c per min and the average rate in LMB with staurosporine was 0.31 n/c per min (Fig. 9A), indicating that in the absence of staurosporine, kinase activity reduced the rate of nuclear influx of Foxo1, presumably by maintaining the concentration of cytoplasmic dephosphorylated Foxo1 at slightly less than half of the concentration attained in the presence of staurosporine. Note that specific inhibition of Akt by Akt-1,2 caused a similar (~2-fold) increase in the rate of Foxo1-GFP nuclear influx in the presence of LMB, confirming that Akt is the predominant kinase phosphorylating cytoplasmic Foxo1 in muscle fibers. As anticipated, IGF-1 treatment completely ablated nuclear influx (Fig. 9B).

First order rate constants for unidirectional cytoplasmic to nuclear fluxes. We next calculate the apparent first order rate constant \(k'\) for unidirectional flux of Foxo from the cytoplasm to the nuclei for the experiment in Fig. 9. With the use of Eq. A4, \(k'\) can be evaluated from successive images acquired at times \(t_1\) and \(t_2\) using the equation:

\[
k' = \frac{2/(c_1 + c_2)\,(n_2 - n_1)/(t_2 - t_1)}{(V_n/V_c)}
\]

where \(n_i\) is the mean pixel fluorescence of the nucleus at a specified time \(t_i\) and \(c_i\) is the mean cytoplasmatic pixel fluorescence at the same specified time. The value used for \(V_n/V_c\) was the mean value of \(-\Delta c/\Delta n\) of 0.049 (±0.0079) obtained from 11 fibers during Foxo1-GFP nuclear influx over time periods sufficient to give relatively large values of \(\Delta c\) (see above). To assess the consistency of \(k'\) during fiber treatment with LMB, we calculated \(k'\) for data values collected at \(t_1\) of 0, 20, 40, 60, 80, and 100 min after LMB addition using the corresponding respective values collected at \(t_2\) of 20, 40, 60, 80, 100, and 120 min after LMB addition. This yielded six consistent \(k'\) values, demonstrating the uniformity of \(k'\) under these conditions (data not shown). Next, we calculated the average \(k'\) for the first 40 min and last 80 min of each group of experiments by averaging the individual \(k'\) calculated per fiber. We then compared these two data sets within each of the three sets of experimental conditions by normalizing all \(k'\) values to the mean \(k'\) of the control period (0–40 min), thus calculating the \(k'\) of the experimental condition (40–120 min) as a fraction of \(k'\) during the control period. The mean \(k'\) in the first 40 min in LMB treatment alone was then compared with the mean \(k'\) for the next 80 min of treatment with LMB and a phosphorylation modulator as a fraction of the LMB alone control period (Fig. 9, C–E). We saw a modest but significant increase in \(k'\) with staurosporine treatment (Fig. 9D) and a more drastic and significant decrease in \(k'\) with IGF-1 (Fig. 9E) whereas fibers treated with LMB alone for the same time showed no differ-
ence in their $k'$ values in the first 40 min compared with the last 80 min (Fig. 9C).

The ratio of the apparent rate constant for nuclear influx during treatment with LMB plus a phosphorylating or a dephosphorylating agent to the rate constant for nuclear influx with LMB alone should be proportional to the fraction of control dephosphorylated Foxo1 that is present in the cytoplasm in the presence of the additional agent, assuming that only unbound dephosphorylated cytoplasmic Foxo1 is imported into the nucleus, that the percent of cytoplasmic dephosphorylated Foxo1-GFP that is not bound to cytoplasmic sites is the same in the two conditions, and that the nuclear transport system itself is not altered by the experimental manipulation. During staurosporine treatment the fraction is 1.4, indicating that general inhibition of phosphorylation by staurosporine caused a 40% increase in the cytosolic concentration of dephosphorylated Foxo1. In sharp contrast, during IGF-1 treatment the fraction is 0.11, indicating a decrease of cytosolic dephosphorylated Foxo1 to 11% of its control level before IGF-1 treatment or a phosphorylation of 89% of the control dephosphorylated Foxo1 on the application of IGF-1. These values, obtained from the kinetic analysis, provide a quantitative measure of the extent to which the inhibition of kinase activity by staurosporine promotes the dephosphorylation of cytoplasmic Foxo1 and IGF-1 promotes its phosphorylation (Fig. 9, C–E).

Fig. 8. Foxo3A cycling and phosphorylation by Akt. A and B: Foxo3A-GFP (red line; $n = 5$ nuclei from 4 fibers) enters the nucleus at a slower rate than does Foxo1-GFP (black line in B; $n = 4$), as determined using LMB to inhibit nuclear efflux. Inhibition of Akt via Akt-I-1,2 (Akt-I) induced an increase in the rate of nuclear influx of both Foxo1-GFP and Foxo3A-GFP. The difference between the relative increases of Foxo1-GFP and Foxo3A-GFP can be seen in B in which the scale is 20-fold that of the same experiment in A. C: representative confocal images of a single muscle fiber expressing Foxo3A-GFP treated with 40 nM LMB with or without Akt-I for the times indicated. Nuclear Foxo3A-GFP increases with LMB treatment revealing nuclear import. With Akt inhibition nuclear import increases indicating cytoplasmic retention to be Akt-dependent. D: unlike the Z-line distribution of Foxo1-GFP (see Fig. 1), Foxo3A-GFP appears as a doublet.

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Akt is necessary for IGF-1-induced cytoplasmic retention of Foxo1. To determine the role of Akt in the reduction of nuclear Foxo1 due to IGF-1 treatment, we determined the rate of nuclear influx during inhibition of Akt in the presence or absence of IGF-1. This experiment had four distinct segments (Fig. 10A). First, a control period of 30 min with no added agents. Second, an 80-min treatment (“0 to 80 min”) with LMB. Third, 40-min (“80 to 120 min”) exposure to 1 μM Akt inhibitor Akt-I-1,2 in the continued presence of LMB. Up to this point all fibers were exposed to the same reagents. Then, in the fourth segment, fibers were treated with or without IGF-1 for 80 min (“120 to 200 min”). Note that media were not changed during the entirety of this experiment and therefore no reagents were removed. The results showed that the rates of nuclear influx with and without IGF-1 in the presence of Akt inhibitor were the same (Fig. 10A), indicating that the entire IGF-1 effect is mediated by Akt and that Akt activity is necessary for IGF-1-induced reduction of nuclear Foxo1. Furthermore, the calculation of the apparent rate constants of cytoplasmic efflux of Foxo1-GFP (k’), as described above, for the last three segments at 0–80, 80–120, and 120–200 min showed IGF-1 treatment to be ineffective in changing the rate constant of cytoplasmic efflux when Akt was inhibited (Fig. 10B). As expected for two samples subjected to the same treatment, the rate constants of nuclear influx from both samples from 0–80 min were not significantly different nor were the rate constants of nuclear influx different for both samples from 80–120 min. Of note, the last time segment did not have significantly different k, regardless of the presence or absence of 100 ng/ml IGF-1, revealing Akt inhibition to be sufficient to fully suppress the IGF-1 effect of reducing nuclear influx of Foxo1.

DISCUSSION

Foxo transcription factors play key roles in cell proliferation, cell cycle, and cellular survival and are expressed in all cells of the human body (1, 13, 23). In skeletal muscle, Foxo proteins play a key role in determining muscle size through the regulation of transcription of atrogene products such as E3 ubiquitin ligases Atrogin-1/MAFbx and MuRF-1 (5, 19, 26).

Here, we utilize confocal imaging of fluorescence from exogenously expressed Foxo1-GFP to monitor kinetics of nuclear-cytoplasmic movements of Foxo proteins in living muscle fibers. Comparison of antibody-stained endogenous Foxo1 and exogenously expressed Foxo1-GFP in terms of sarcomeric localization (Fig. 1), nuclear-cytoplasmic distribution (Fig. 2A), and response to treatment (Fig. 4, E and F) validate this system of adenovirally expressed Foxo1-GFP as a reliable indicator of endogenous Foxo and as a useful tool in measuring rates of nuclear-cytoplasmic translocation in studies involving fluorescently tagged Foxo.

Our results with Foxo1-GFP show that IGF-1 treatment alone is sufficient to prevent nuclear targeting of Foxo1 in live
together with the signaling systems and inhibitors that we have
micro movements of Foxo proteins is presented in Fig. 11,
activity, as well as its role in nuclear-cytoplasmic cycling of
cytoplasm but does reveal the importance of its cytoplasmic
). This does not limit PP2A’s role to the
functional role in Foxo1 dephosphorylation in skeletal muscle,
inhibition of PP2A via OA (Fig. 5) demonstrates PP2A’s
impressive decrease in nuclear Foxo1 in response to
(37). The impressive decrease in nuclear Foxo1 in regulation of its rate of cytoplasmic efflux (Figs. 6,
9, and 10). Furthermore, the increase in the rate of nuclear
influx that resulted from staurosporine addition in the presence
of LMB (Fig. 9A) indicates that the nuclear import machinery
is not saturated at the level of expression of Foxo1-GFP
employed under our conditions of infection by adFoxo1-GFP.
Based on the important information obtained here and else-
where with the nuclear export inhibitor LMB, identification of
a comparable specific inhibitor of nuclear import would further
open up the field to understanding the kinases and phospho-
tases that regulate Foxo1 nuclear export.

The nuclear export of Foxo can be inhibited by LMB (Fig.
11), which binds to and thus removes the availability of CRM1
for nuclear export. In the presence of a fully blocking
concentration of LMB, any Foxo that enters the nucleus is unable to leave and becomes trapped in the nucleus. Inhibition of nuclear export via LMB thus provides a powerful tool for measuring the rate of unidirectional nuclear influx and for calculating its rate constant of cytoplasmic efflux. The change in the rate constant for unidirectional efflux out of the cytoplasm due to treatment with phosphorylation modulators demonstrates the importance of cytoplasmic phosphorylation/dephosphorylation of Foxo1 in regulation of its rate of cytoplasmic efflux (Figs. 6,
9, and 10). Furthermore, the increase in the rate of nuclear
influx that resulted from staurosporine addition in the presence
of LMB (Fig. 9A) indicates that the nuclear import machinery
is not saturated at the level of expression of Foxo1-GFP
employed under our conditions of infection by adFoxo1-GFP.
Based on the important information obtained here and else-
where with the nuclear export inhibitor LMB, identification of
a comparable specific inhibitor of nuclear import would further
open up the field to understanding the kinases and phospho-
tases that regulate Foxo1 nuclear export.

It should be noted that the same unidirectional flux of Foxo
from muscle fiber nuclei can be considered as either a unidi-
rectional efflux out of the cytoplasm or as a unidirectional
influx into the nuclei. In practical terms of ease of experimental
measurement, it is more convenient to monitor the rate of
change of Foxo-GFP fluorescence in the nuclei than in the
cytoplasm. The total volume of the nuclei is much smaller than
that of the cytoplasm, so the corresponding change in mean
pixel fluorescence for a given flux of Foxo-GFP between
cytoplasm and nuclei is much larger in the nuclei than in the
cytoplasm. By conservation of mass, the ratio of changes of
nuclear to cytoplasmic mean pixel fluorescence for a given
movement of Foxo-GFP between nuclei and cytoplasm is equal
to the ratio of cytoplasmic to nuclear volume in the muscle
fibers. However, in terms of mechanistic interpretation of
the nuclear import system, it is more appropriate to consider
cytoplasmic rather than nuclear concentration change and the
rate of efflux of Foxo out of the cytoplasm. This is because the
unidirectional flux of Foxo from cytoplasm to nuclei is deter-
mined by the cytoplasmic concentration of dephosphorylated
unbound Foxo and is independent of the nuclear concentration,
as indicated in the kinetic scheme in Fig. 11 and by Eq. A1.
The unidirectional rate constant of cytoplasmic efflux of Foxo1 is relatively high compared with that of other transcription factors that our laboratory has studied. Based on the nuclear influx measured in the presence of LMB, the apparent first order rate constant $k'$ for unidirectional flux of Foxo1-GFP out of the cytoplasm and into the nuclei was $0.074 \pm 0.005$ per hour under resting conditions (data from Fig. 10). In contrast, under resting conditions, the transcription factor NFATc1 leaves the cytoplasm and enters the nucleus at a much slower rate. As determined in LMB, the unidirectional flux of NFATc1 from cytoplasm to nucleus occurs with an apparent first order rate constant of $0.035 \pm 0.005$ per hour [based on data used to make Figs. 2C and 6C in Shen et al. (30), assuming $V_n/V_c$ is the same for Foxo1 and NFATc1]. Foxo3A also enters the nucleus much more slowly than Foxo1. The unidirectional first order apparent rate constant for movement of Foxo3A out of the cytoplasm and into the nucleus is $0.023 \pm 0.004$ per hour in the presence of LMB (calculated from the data for nuclei in fibers in Fig. 8). One possible explanation for the lower unidirectional apparent rate constants for movement of NFATc1 and Foxo3A out of the cytoplasm compared with the rate constant for Foxo1 could be their less effective transport by the nuclear import system, i.e., the actual rate constant $k$ would be considerably lower for Foxo3A or NFATc1 than that for Foxo1, but this may be unlikely for the similar molecules Foxo1 and Foxo3A. Alternatively, the fractional dephosphorylation of NFATc1 or Foxo3A in the cytoplasm might be much higher than that of Foxo1. A much lower relative degree of dephosphorylation of Foxo3A than Foxo1 in muscle fibers would be consistent with the observation that nerve growth factor activated Foxo phosphorylation in PC12 cells occurs at considerably lower nerve growth factor levels for Foxo3 than for Foxo1(35). Finally, the fraction of unbound dephosphorylated Foxo1 in the cytoplasm could be considerably greater than the fraction of unbound dephosphorylated Foxo3A or NFATc1 in the cytoplasm. Intriguingly, the sub-sarcomeric distribution pattern for Foxo3A is different from that of Foxo1, possibly indicative of a difference in binding and fraction bound. The presence of two Foxo isoforms, Foxo1 and 3A, having ~20-fold different nuclear-cytoplasmic shuttling rates under control conditions, but presumably modulating the expression of the same group of genes, raises interesting questions regarding mechanism, regulation, and function that merit future investigation of Foxo isoforms.

**APPENDIX**

**Apparent first order rate constant for unidirectional flux of Foxo from cytoplasm to nuclei.** Assuming that the rate of nuclear efflux is 0 with LMB treatment, that the movement of Foxo-GFP out of the cytoplasm is a first order process, and that only dephosphorylated and unbound Foxo-GFP can enter the nucleus, the rate of change of cytoplasmic Foxo-GFP fluorescence due to movement of Foxo-GFP out of the cytoplasm is given by:

$$\frac{dc}{dt} = -k f_c c,$$  \hspace{1cm}  \text{(A1)}

where $c$ is the mean pixel fluorescence in the cytoplasm, $t$ is time, $k$ is the first order rate constant for movement of dephosphorylated and unbound Foxo-GFP from the cytoplasm to the nucleus, and $f_c$ is the fraction of total cytoplasmic Foxo-GFP that is both dephosphorylated and unbound. Defining the apparent rate constant $k'$ as $k f_c$, $k'$ is given by

$$k' = -\left(1/f_c\right)(dc/dt).$$  \hspace{1cm}  \text{(A2)}

Rearranging the equation for conservation of mass (Eq. 1) and taking the time derivative gives

$$\frac{dc}{dt} = -\left(\frac{dn}{dt}\right)(V_n/V_c).$$  \hspace{1cm}  \text{(A3)}

Substitution of Eq. A3 into Eq. A2 gives the equation

$$k' = \left(1/f_c\right)(dn/dt)(V_n/V_c).$$  \hspace{1cm}  \text{(A4)}

for the apparent first order rate constant for Foxo-GFP movement out of the cytoplasm and into the nuclei.
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