Number and Brightness Analysis of LRRK2 Oligomerization in Live Cells

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ABSTRACT Leucine-rich repeat kinase 2 (LRRK2) is a large multidomain protein that contains enzymatically functional GTPase and kinase domains. Several noncoding LRRK2 gene polymorphisms have been associated with susceptibility to Parkinson's disease (PD), Crohn's disease, and leprosy. Many LRRK2 coding polymorphisms have been associated with or causally linked to PD. The G2019S point mutation within the LRRK2 kinase domain is the most common cause of familial PD. The G2019S mutation appears to alter LRRK2 kinase activity. Some but not all studies have reported that LRRK2 kinase activity is dependent upon LRRK2 dimerization and membrane localization. It is important to define the oligomeric state(s) of LRRK2 in living cells, which to date have only been characterized in vitro. Here we use confocal and total internal reflection microscopy coupled with number and brightness analysis to study the oligomeric states of LRRK2 within the cytosol and on the plasma membrane of live CHO-K1 cells. Our results show, for the first time to our knowledge, that LRRK2 is predominantly monomeric throughout the cytosol of living cells, but attains predominately higher oligomeric states in the plasma membrane.

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Leucine-rich repeat kinase 2 (LRRK2) is a large (2527 amino acids) multidomain protein that contains enzymatically functional GTPase and kinase domains (1–6). Missense point mutations in LRRK2 are the most common cause of familial Parkinson's disease (PD) (7). The molecular mechanisms by which LRRK2 mutations cause PD remain uncertain. In vitro studies are consistent with a toxic gain-of-function mechanism that is dependent upon LRRK2 kinase activity (1–5). This possibility suggests that LRRK2-specific kinase inhibitors may have therapeutic benefit. LRRK2 kinase activity has been reported to be dependent upon LRRK2 dimerization (8) and inhibiting LRRK2 dimerization may be a good alternative therapeutic approach. However, Ito and Iwatsubo (9) have recently reported that LRRK2 is predominantly monomeric and that dimerization is dispensable for LRRK2 enzymatic activity. Because previous studies have analyzed LRRK2 in vitro, it is important to determine the oligomeric state(s) of LRRK2 in living cells, which remains a key unanswered question.

Evidence for potential LRRK2 oligomerization was initially obtained using tandem affinity purification (10) as well as by yeast two-hybrid analysis and by coprecipitation of differentially tagged LRRK2 fragments from transfected cells (11). Subsequent analyses of LRRK2 oligomerization have employed classical biochemical techniques. For example, Greggio et al. (11) and Sen et al. (8) estimated that LRRK2 is primarily dimeric, based on size-exclusion chromatography and native PAGE extracts from LRRK2 overexpressing cells. Berger et al. (12) used a combination of techniques, including density gradient centrifugation, size-exclusion chromatography, and native gel electrophoresis, to demonstrate that LRRK2 extracted from cell membranes is both monomeric and dimeric, whereas cytosolic fractions contain almost exclusively monomeric LRRK2.

By contrast, Jorgensen et al. (13) found no evidence of LRRK2 monomer in cell lysates analyzed by size exclusion chromatography and blue native gel electrophoresis. However, as mentioned, Ito and Iwatsubo (9) reported, based on density gradient centrifugation, that LRRK2 is predominantly monomeric. Although these studies have provided valuable information regarding LRRK2 self-association and its potential physiological significance, they have relied exclusively on in vitro analyses of cell extracts, which may account for some of the conflicting conclusions. Here we apply number and brightness (N&B) analysis of fluorescence fluctuation spectroscopy data to directly measure LRRK2 oligomerization in the cytoplasm and membranes of living cells.

CHO-K1 cells were transfected using a pCMV vector (Clontech, Mountain View, CA) engineered to express EGFP fused to the N-terminus of wild-type mouse LRRK2. The EGFP coding sequence contained an A207K

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mutation to eliminate potential dimerization of the EGFP moiety. Confocal images were collected 24 h after transfection, using a Fluoview 1000 microscope (Olympus, Melville, NY) in the photon-counting mode, with EGFP excitation from the 488-nm line of an Argon-ion laser (scan speed 12.5 μs/pixel, scan area 256 $\times$ 256 pixels). Total internal reflection fluorescence (TIRF) measurements were conducted with an instrument previously described by Unruh and Gratton (14) and Ross et al. (15) using 50-ms exposure time per frame. N&B analysis allows for the determination of the number (N) of diffusing particles within the focal spot and the intrinsic brightness (B) of each particle,

$$N = \frac{(\langle I\rangle - offset)^2}{\sigma^2 - \sigma_0^2}, \quad B = \frac{\sigma^2 - \sigma_0^2}{\langle I\rangle - offset}$$

where I is the signal intensity, σ^2 is the variance, and *offset* and ${\sigma_0}^2$ are the intensity offset and readout noise variance of the detection electronics, respectively. N&B provides a map of number and brightness for every pixel in the image by calculating the average and variance of the intensity distribution in each pixel (16,17).

In the TIRF system, with an analog camera, both the offset and gain must be calibrated in terms of the digital level/counts (14). A separate set of CHO-K1 cells transfected with monomeric EGFP and monomeric EGFP fused to the membrane targeting sequence GAP-43 were used as the brightness standards for confocal and TIRF experiments, respectively.

Fig. 1 *A* shows a representative image of a CHO-K1 cell transfected with EGFP-LRRK2. The focal plane was positioned just above the nucleus, as EGFP-LRRK2 is not found in the nucleus (data not shown). The fluorescence intensity levels varied throughout the cell, indicative of LRRK2 localization. Using N&B analysis (Fig. 1 *B*), and normalizing the brightness levels using the monomeric EGFP standard (B value = 1.09), one finds that the green pixels correspond to the brightness values of monomeric EGFP while the red pixels correspond to brightness levels of approximately three times the monomeric level. The red pixels may represent a combination of dimer and tetramer or some other combination of higher oligomers. The brightness plot shown in Fig. 1 *C* indicates that much of the cytosolic EGFP-LRRK2 was present in the monomeric state (*green pixels* in Fig. 1 *C*) whereas most of the higher-order oligomeric states (*red pixels* in Fig. 1 *C*) were found near the cell periphery (~84% of the pixels, analyzed from two cells, corresponded to monomeric EGFP-LRRK2).

The presence of higher oligomeric states in the plasma membrane of the cells was verified using TIRF microscopy (Fig. 2). The density of EGFP-LRRK2 varied in the plasma membrane, with some distinct areas of more concentrated LRRK2. N&B analysis reveals that the oligomeric state of EGFP-LRRK2 was heterogeneous on the plasma membrane, though it was predominately oligomeric (~80% of the pixels analyzed, from two cells, corresponded to oligomeric states greater than monomer). In Fig. 2 *C* the green pixels correspond to monomeric brightness levels (B value = 1.06 for standard) while the red and blue pixels correspond to dimer brightness levels and higher-order oligomers, respectively. We note that the pixels with very low intensities surrounding the cell were not included because these were judged to correspond to parts of the plasma membrane that were not adhering to the surface, i.e., which were raised slightly off the substrate, and hence in a weaker portion of the illumination field.

Our confocal data demonstrate that the oligomeric state of LRRK2 in living cells depends on the subcellular localization. We observed a distribution of predominately LRRK2 monomers in the cytosol whereas the confocal/TIRF analysis is consistent with a distribution of predominately higher-order (e.g., dimers and tetramers) oligomeric states of LRRK2 on the plasma membrane. Biochemical studies have shown that the dimer form of LRRK2 has greater kinase activity than monomeric LRRK2 and that membrane fractions of cell lysates are enriched with LRRK2 dimer

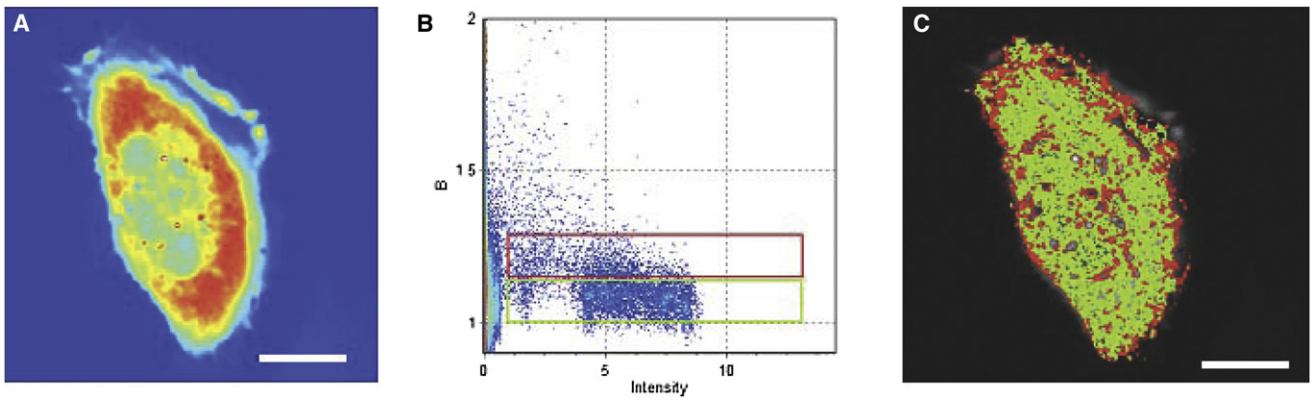

FIGURE 1 (*A*) Confocal intensity image of EGFP-LRRK2. (*B*) Brightness versus intensity plot showing the monomeric LRRK2 (*green box*) and higher-order oligomers (*red box*). (*C*) Brightness map highlighting the regions within the cells containing monomer (*green*) and higher-order oligomers (*red*). Bars equal 15 μm.

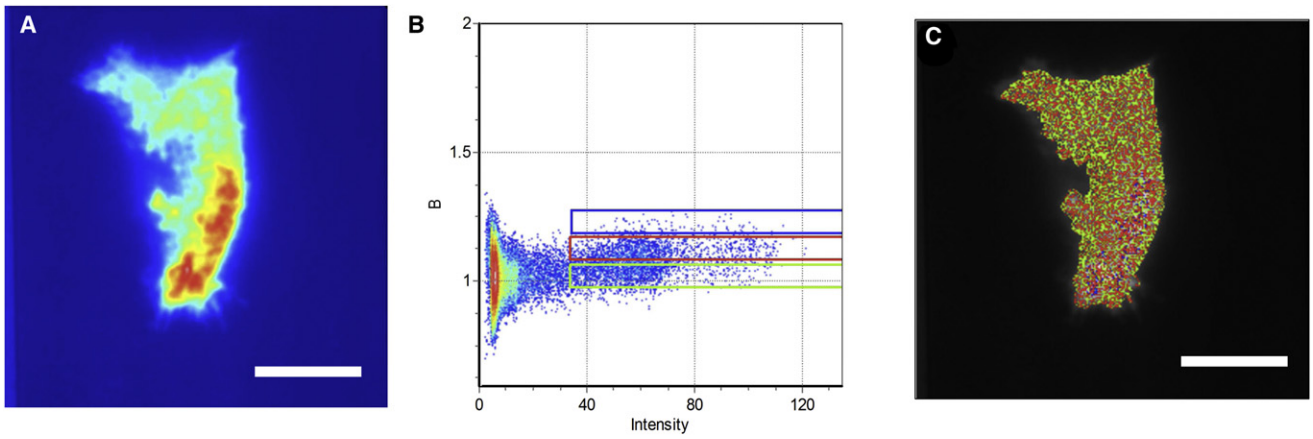

FIGURE 2 (*A*) TIRF intensity image of EGFP-LRRK2. (*B*) Brightness-versus-intensity plot showing the monomeric LRRK2 (*green box*), dimeric LRRK2 (*red box*), and the higher-order oligomers (*blue box*). (*C*) Brightness map highlighting the regions containing monomer (*green*), dimer (*red*), and higher-order oligomers (*blue*). Bars equal 17 μm.

(6,12). These data and our N&B analysis support a model of LRRK2 in which the protein cycles between a low-activity monomeric state and high-activity multimeric (dimer and/or higher) states. Predominantly monomeric LRRK2 in the cytosol can be recruited to the plasma membrane, either directly or indirectly, through a regulatory mechanism yet defined. Association with the plasma membrane increases local concentration and presumably induces self-association.

The use of in vivo fluorescence fluctuation analysis allows us to address the oligomeric states of the PD-linked protein LRRK2 in living cells. In particular, we demonstrate for the first time (to our knowledge) that the LRRK2 protein can exist in oligomeric forms on the plasma membrane of living cells.

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