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Molecular Brain Research 123 (2004) 27–36
MOLECULAR BRAIN RESEARCH
www.elsevier.com/locate/molbrainres

Research report

Retrograde transport of the glucocorticoid receptor in neurites requires dynamic assembly of complexes with the protein chaperone hsp90 and is linked to the CHIP component of the machinery for proteasomal degradation

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Accepted 24 December 2003

Abstract

Here, we have used a chimera of green fluorescent protein (GFP) and the glucocorticoid receptor (GR) to study retrograde movement of a model soluble (i.e., non-vesicle-associated) protein in axons and dendrites of cultured NT2-N neurons. It is known that in non-neuronal cells, the GFP–GR moves from cytoplasm to the nucleus in a steroid-dependent manner by a rapid, hsp90-dependent mechanism. When rapid movement is inhibited by geldanamycin (GA), a specific inhibitor of the protein chaperone hsp90, the GFP–GR translocates slowly to the nucleus by diffusion. Here we show that GFP–GR expressed in hormone-free neurons is localized in both cytoplasm and neurites, and upon treatment with dexamethasone (DEX), it moves to the nucleus. In neurites, movement by diffusion is not possible, and we show that movement of the GFP–GR from neurites is blocked by geldanamycin, suggesting that the hsp90-dependent movement machinery is required for retrograde movement. In cells treated with both dexamethasone and geldanamycin, the GFP–GR becomes concentrated in fluorescent globules located periodically along the neurites. Carboxyl terminus of Hsc70-interacting protein (CHIP), the E3 ubiquitin ligase for the GR, also concentrates in the same loci in a steroid-dependent and geldanamycin-dependent manner. If geldanamycin is removed, the GFP–GR exits the globules and continues its retrograde movement. However, in the continued presence of geldanamycin, the GFP–GR in the globules undergoes proteasomal degradation, suggesting that the globules function as degradasomes. This is the first evidence for a linkage between receptor trafficking along neurites and receptor degradation by the proteasome.
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Theme: Cellular and molecular biology
Theme: Cytoskeleton transport and membrane targeting

Keywords: Protein trafficking; hsp90; Proteasomal degradation; Glucocorticoid receptor; CHIP; Geldanamycin

1. Introduction

Although much is known about the movement of vesicles and organelles in anterograde and retrograde directions along axons [12], very little is known about the transport of soluble proteins (i.e. non-vesicular proteins) in axons and dendrites. Axons and dendrites are specialized cytoplasmic extensions where movement by random diffusion alone would not permit delivery of protein solutes over long distances, and a machinery for movement is required. The glucocorticoid receptor (GR) is a soluble protein that moves rapidly from the cytoplasm to the nucleus in a hormone-dependent manner [20], and here we use a transcriptionally active chimera of the green fluorescent protein (GFP) and the GR to study receptor movement in axons and dendrites of human NT2-N neurons.

Abbreviations: GR, glucocorticoid receptor; GFP, green fluorescent protein; hsp, heat shock protein; TPR, tetratricopeptide repeat; CHIP, carboxyl terminus of Hsc70-interacting protein; HOP, hsp70/hsp90 organizing protein; DEX, dexamethasone; GA, geldanamycin

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doi:10.1016/j.molbrainres.2003.12.015

In this study, no distinction can be made between axons and dendrites, and we will use the term neurite to refer collectively to both axons and dendrites.

Previous studies in mouse L929 and 3T3 fibroblasts have shown that cytoplasmic-nuclear translocation of the endogenous GR [6] or GFP–GR [8,9] is inhibited by geldanamycin, an ansamycin antibiotic that binds to the N-terminal ATP site of hsp90 and inhibits its function [22]. Rapid translocation of the GR in the absence of geldanamycin ($t1/2 \sim 5$min) requires intact cytoskeleton, and geldanamycin slows ($t1/2 \sim 45$min) but does not block translocation [8,10]. Thus, in the non-neuronal cell, there is a rapid, hsp90-dependent (geldanamycin-inhibited) movement along cytoskeletal tracts, but when that mechanism is inhibited, slow movement occurs by diffusion.

It has been shown that retrograde transport of vesicles [15,26,27] in axons requires cytoplasmic dynein, a molecular motor that processes along microtubular tracks towards the minus ends [25]. Microinjection studies in axons show that protein solutes containing a nuclear localization signal utilize a microtubule-based machinery for retrograde movement [1]. Although it seems clear that cytoplasmic dynein is the motor for retrograde movement in axons, it is not known how the motor recognizes its cargo [12].

For retrograde movement of the GR in non-neuronal cells, the linkage to cytoplasmic dynein has been established. GR·hsp90 heterocomplexes isolated from cytosol or assembled in reticulocyte lysate contain an immunophilin, such as FKBP52, and cytoplasmic dynein [10]. GR·hsp90 heterocomplexes are assembled by an ubiquitous, multiprotein, hsp90/hsp70-based chaperone machinery, and after assembly, an immunophilin binds via its tetratricopeptide repeat (TPR) domain to an acceptor site on hsp90 [19]. The hsp90-binding immunophilins also possess a peptidylprolyl isomerase (PPIase) domain that functions as a protein interaction domain to link them to cytoplasmic dynein [11,23]. Competition with a PPIase domain fragment disengages the GR·hsp90·immunophilin complex from dynein in vitro, and expression of the fragment inhibits rapid translocation of the GFP–GR in vivo, much like treatment with geldanamycin [10].

In this paper, we present evidence that this hsp90-dependent movement system is responsible for retrograde, steroid-dependent movement of GFP–GR in neurites of NT2-N neurons, which are derived from the human NT2 teratocarcinoma cell line by inducing a postmitotic neuronal phenotype with retinoic acid [2,17,18]. As shown previously in fibroblasts, geldanamycin slows, but does not block, retrograde movement of GFP–GR in the NT2-N cell body. In NT2-N neurites, GFP–GR movement is blocked by geldanamycin, and the receptor collects in fluorescent globules located periodically along the neurites. If geldanamycin is withdrawn, the GFP–GR exits the globules and movement continues, but in the continued presence of geldanamycin, the GFP–GR is degraded by the proteasome. Carboxyl terminus of Hsc70-interacting protein (CHIP) [13], which is the E3 ubiquitin ligase for the GR [4], also moves into the globules by a glucocorticoid-dependent mechanism when cells are treated with geldanamycin. Thus, in the neurites of cells producing large amounts of the receptor chimera, the ubiquitin ligase that initiates the process of receptor degradation moves with the receptor into centers where receptor degradation occurs in the continued presence of geldanamycin.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were from BioWhittaker (Walkersville, MD), and Opti-MEM I medium was from Gibco (Grand Island, NY). Charcoal-stripped calf serum was from Sigma (St. Louis, MO), and the Trans-Fast kit for cell transfection was from Promega (Madison, WI). Matrigel basement membrane matrix and poly-D-lysine hydrobromide were from Collaborative Biomedical Products (Bedford, MA). The proteasome inhibitor MG-132 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA), and the hsp90 inhibitor geldanamycin was obtained from the Drug Synthesis and Chemistry branch of the Developmental Therapeutics Program, National Cancer Institute. Construction of the GFP–GR was described previously [8], as was preparation of the rabbit anti-CHIP antibody [3]. The BB70 mouse monoclonal IgG against hsp70 was from StressGen (Victoria, BC), and the 3G3 mouse monoclonal IgM against hsp90 was from Affinity BioReagents (Golden, CO). Rhodamine-conjugated donkey anti-rabbit IgG, anti-mouse IgG and anti-mouse IgM were from Jackson ImmunoResearch (West Grove, PA).

2.2. Cell culture and transfection

NT2 stem cells (passages 50–80) were grown in 75-cm^2 tissue culture flasks containing DMEM supplemented with 10% fetal calf serum. NT2 cells were differentiated into the neuronal phenotype, NT2-N, as described previously [18] and modified by Novak et al. [17]. Briefly, NT2 cells were treated with 10 μM retinoic acid twice per week for 5 weeks. After retinoic acid treatment, cells were rinsed with phosphate-buffered saline containing 0.2 mg/ml EDTA, detached from culture flasks with 0.25% (w/v) trypsin, and replated (replate I). Two days later, cells were rinsed with Hank's balanced salt solution and treated with 0.05% trypsin/0.53 mM EDTA. Flasks were then struck repeatedly to remove NT2-N cells, which were replated on coverslips previously coated with 10 μg/ml poly-D-lysine and 1:36 Matrigel in DMEM (replate II). NT2-N cells were seeded at a density of $2.5–5.0 \times 10^6$ cells/dish. For the first 3 weeks after replate II, NT2-N cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin G, 100 μg/ml

streptomycin sulfate, 100 μM 5-fluoro-2-deoxyuridine, 10 μM uridine, and 1 μM cytosine β-D-arabinofuranoside. After 3 weeks, NT2-N cells were maintained in conditioned medium obtained from replate I. NT2-N cells were used for experiments 6 to 8 weeks after replate II. The conditioned medium was replaced by Opti-MEM supplemented with 10% fetal calf serum, and after 3 h, this medium was replaced by Opti-MEM only. Cells were transfected with 3.5 μg/ml of GFP–GR plasmid preincubated for 10 min with 3.0 μl of Trans-Fast reagent per μg of DNA. After 2 h, the transfection medium was aspirated and replaced with fresh Opti-MEM with 10% fetal calf serum. After 24 h, the medium was removed, cells were washed four times with Opti-MEM medium, and incubated for an additional 24 h in Opti-MEM with 10% charcoal-stripped calf serum.

2.3. GFP–GR translocation

The transfected cells on coverslips were incubated with 1 μM dexamethasone, and at various times, coverslips were removed, rinsed with ice-cold phosphate-buffered saline, and fixed by immersion in −20 °C methanol for at least 15 min. Cells were rinsed again with phosphate-buffered saline, and the coverslips were inverted onto a slide with 5 μl of mounting solution (1 mg/ml *p*-phenylenediamine in 10% phosphate-buffered saline, 90% glycerol, pH 9.0). Cells were visualized with a Leitz Aristoplan epiillumination microscope.

Cells were scored for GFP–GR translocation to the nucleus as described previously [8], using a score of 4 for nuclear fluorescence much greater than cytoplasmic fluorescence, 3 for nuclear fluorescence greater than cytoplasmic fluorescence, 2 for nuclear fluorescence equal to cytoplasmic fluorescence, 1 for nuclear fluorescence less than cytoplasmic fluorescence, and 0 for nuclear fluorescence much less than cytoplasmic fluorescence. The translocation scores represent the means ± S.E. from three experiments in which >50 cells per condition per experiment were scored.

Cells treated with dexamethasone and geldanamycin developed globules of fluorescence in their neurites. These fluorescent globules are readily differentiated from much smaller and more intense speckles of fluorescence that are seen occasionally in cells overexpressing the GFP–GR, regardless of treatment condition. To score the globule formation, those cells that showed at least three globules per neurite were expressed as a percent of the total number of cells exhibiting fluorescent neurites.

2.4. Geldanamycin treatment

To assay the effect of the hsp90 inhibitor, geldanamycin, on GFP–GR movement in NT2-N cells, dexamethasone (1 μM) or vehicle (0.1% ethanol) was added to cultures precooled for 10 min on ice. Cells were maintained on ice for 1.5 h to allow steroid binding to the receptor, which is located in the cytoplasm and axoplasm. Geldanamycin (10 μM) or vehicle (0.1% DMSO) was then added, and the incubation was continued on ice for 30 min. At the end of this preincubation, the cold medium was replaced with warm (37 °C) medium containing the same components, and the incubation was continued at 37 °C to permit nuclear translocation. At the indicated times, the cells were fixed in cold methanol and GFP–GR translocation was scored.

2.5. Washout experiments

NT2-N cells expressing GFP–GR were pretreated at 0 °C with dexamethasone and geldanamycin as described above, and the cells were then incubated at 37 °C for 1 h. The medium was aspirated, the cells were washed twice with Opti-MEM, and the washed cells were incubated for additional times up to 3 h at 37 °C in Opti-MEM/10% charcoal-stripped calf serum in the presence or absence of 10 μM geldanamycin and/or 10 μM of the proteasome inhibitor MG-132. The coverslips were then withdrawn from the medium, rinsed with phosphate-buffered saline, and fixed in −20 °C methanol as described above.

2.6. Indirect immunofluorescence

NT2-N cells expressing GFP–GR were pretreated with 10 μM geldanamycin, or 1 μM dexamethasone, or both as described above. After 1 h of incubation at 37 °C, the cells were rinsed with phosphate-buffered saline and fixed in cold (−20 °C) methanol for 10 min. Then, coverslips were inverted onto 100 μl of blocking buffer (20 mM Tris at pH 8.8, 0.63 M NaCl, 0.05% Tween-20, 0.02% NaN_3 and 1% BSA) containing 1 μl of anti-CHIP antibody, 2 μl BB70 IgG against hsp70, 3 μl 3G3 mouse IgM against hsp90, or 2 μl UPJ56 rabbit anti-FKBP52 serum. After overnight incubation at 4 °C, coverslips were washed with blocking buffer for 15 min at room temperature and inverted onto 100 μl of blocking buffer containing 2 μl of rhodamine-conjugated donkey anti-rabbit IgG, or anti-mouse IgG, or anti-mouse IgM. After 2 h at room temperature, the cells were washed, mounted on microscope slides and visualized.

3. Results

3.1. GFP–GR movement is inhibited by geldanamycin

To determine if GFP–GR distributes to neurites and undergoes retrograde movement, NT2-N cells were transfected with GFP–GR plasmid, and 48 h later, they were treated with dexamethasone (Fig. 1). As can be seen in the cells that were not treated with dexamethasone (−DEX condition in Fig. 1), GFP–GR was localized in the cytoplasm with extension into NT2-N neurites, and this distribution was not affected by 60 min of treatment with geldanamycin. In the absence of geldanamycin, treatment with dexamethasone

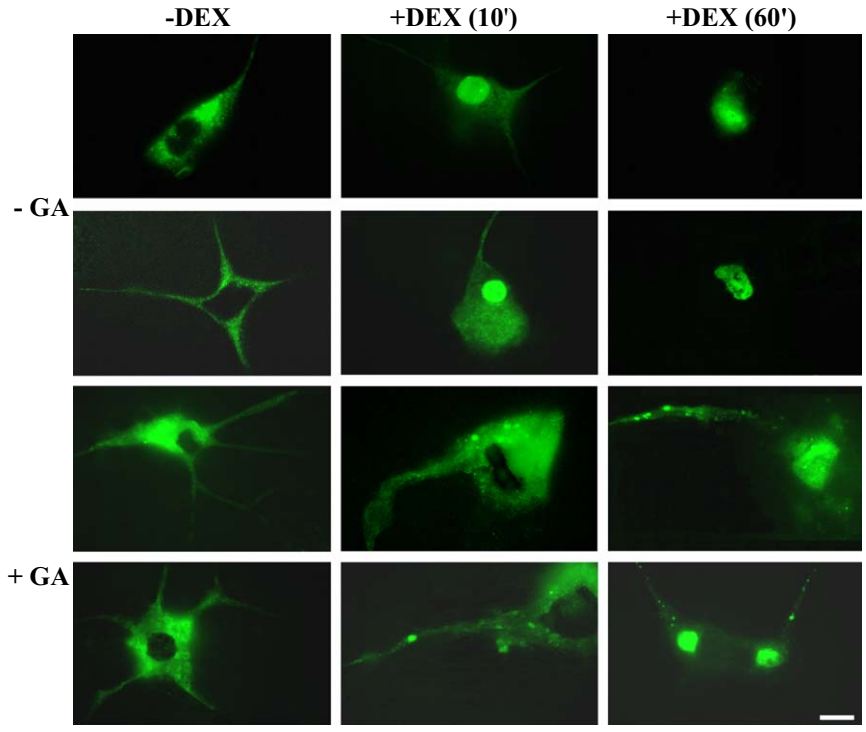

Fig. 1. Geldanamycin inhibits GFP–GR movement in neurites. NT2-N cells transfected with GFP–GR were preincubated on ice with 1 μM dexamethasone (DEX) and/or 10 μM geldanamycin (GA) as described under Materials and methods. The cells were then incubated for 10 or 60 min at 37 °C to permit GFP–GR translocation to the nucleus. Two cells are shown for each condition. Cells that were not treated with dexamethasone were incubated for 60 min with vehicle at 37 °C prior to fixation. The bar at the lower right represents 10 μm.

caused movement of the GFP–GR to the nucleus, with nuclear accumulation being clearly evident after 10 min and complete by 60 min. In cells treated with dexamethasone and geldanamycin, there was little nuclear accumulation at 10 min, but by 60 min, much of the GFP–GR had translocated to the nuclei. However, in contrast to cells that were treated with dexamethasone in the absence of geldanamycin, green fluorescence was still present in neurites of cells treated for 60 min with dexamethasone in the presence of geldanamycin. It should be noted that the receptors were first bound with steroid while the cells were maintained on ice and then exposed to geldanamycin when the temperature was raised to 37 °C to permit receptor movement. This avoids any geldanamycin-mediated decrease in the steroid binding activity of unliganded GFP–GR.

We have shown previously that geldanamycin slows the rate of, but does not block, GFP–GR translocation from the cytoplasm to the nucleus of NIH 3T3 mouse embryo fibroblasts [8,10]. In Fig. 2, we have scored the dexamethasone-dependent translocation of the GFP–GR from the cytoplasm to the nucleus of NT2-N cells in the presence and absence of geldanamycin treatment. As shown in Fig. 1, cells treated with dexamethasone and geldanamycin for 60 min have immunofluorescence remaining in neurites when most of the GFP–GR in the cell body has translocated to the nucleus. For scoring the translocation in Fig. 2, the relative GFP–GR fluorescence in cell bodies and nuclei were compared, and GFP–GR fluorescence in neurites was ignored. In the absence of geldanamycin, the GFP–GR rapidly translocated to the nucleus (open circles, $t1/2 \sim 10$ min), but when hsp90 function was inhibited by

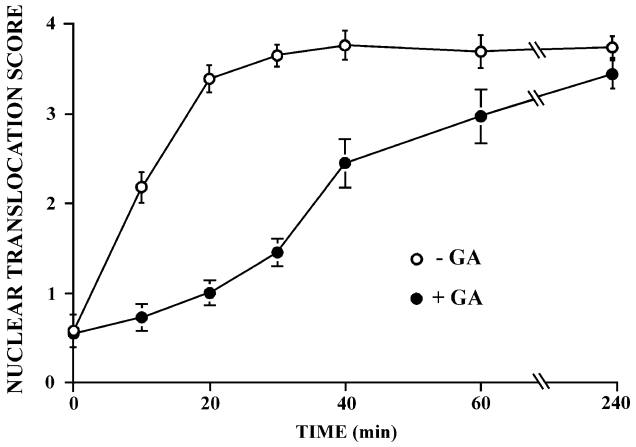

Fig. 2. Geldanamycin slows GFP–GR translocation in the cell body from the cytoplasm to the nucleus. NT2-N cells expressing GFP–GR were preincubated on ice with 1 μM dexamethasone and either DMSO vehicle (○) or 10 μM geldanamycin (●). The temperature was shifted to 37 °C, and at various times, cells were fixed and scored for GFP–GR translocation from the cell body (i.e., not including neurites) to the nucleus. Each value represents the translocation score (mean ± S.E.) of three independent experiments.

geldanamycin (solid circles), the translocation rate was much slower.

3.2. GFP–GR movement in neurites

The slowing of GFP–GR movement in the cell body by geldanamycin suggests that there is a rapid, hsp90-dependent movement mechanism, and when hsp90 is inhibited by geldanamycin, the GFP–GR translocates slowly in a manner that may be diffusion-limited in the cytoplasm. Because movement by diffusion is not an alternative in neurites, we asked whether geldanamycin would block GFP–GR movement in neurites. Only 50–60% of NT2-N cells expressing GFP–GR have visible fluorescence in neurites, but when cells are treated with dexamethasone, the percentage of cells with fluorescence in neurites decreased in a time-dependent manner (Fig. 3, open circles). As can be seen from the open circles in Fig. 3, the rate at which cells lost their neurite fluorescence is slower ($t1/2$ 30–40 min) than the rate at which fluorescence shifted in the cell body (Fig. 2) from the cytoplasm to the nucleus ($t1/2$ ~ 10 min). This slower rate of fluorescence depletion in neurites is consistent with a longer travel distance. However, by 60 min of dexamethasone treatment, most of the GFP–GR had moved out of the neurites. In cells treated with geldanamycin, there was no loss of neurite fluorescence over 60 min of dexamethasone treatment (Fig. 3, solid circles), suggesting that GFP–GR movement in neurites is entirely hsp90-dependent.

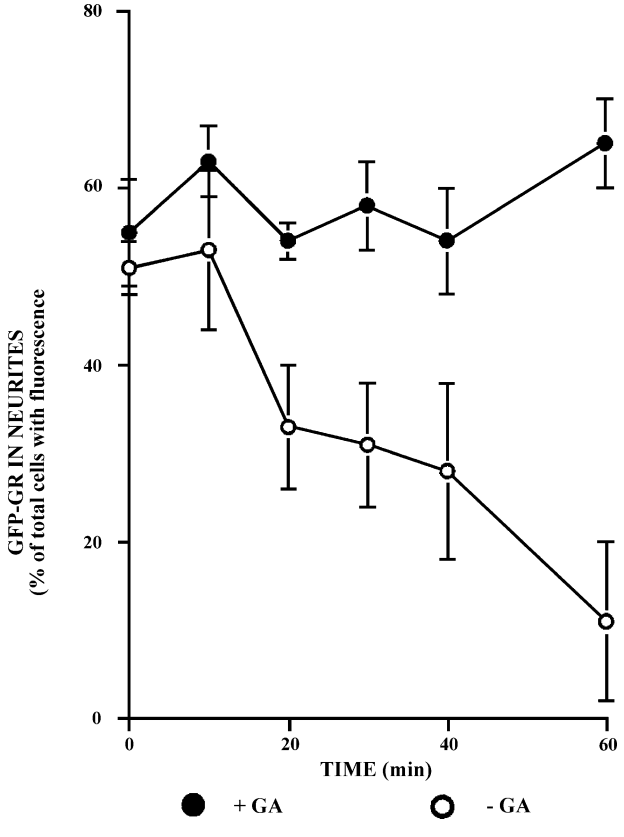

Fig. 3. Geldanamycin blocks GFP–GR movement in neurites. NT2-N cells expressing GFP–GR were pretreated on ice with 1 μM dexamethasone in the absence (open circles) or presence (solid circles) of 10 μM geldanamycin. The temperature was shifted to 37 °C (zero time), and fluorescence in neurites was scored. To determine GFP–GR in neurites, the number of cells with one or more fluorescent neurites was scored as a percent of the total number of fluorescent cells. Each value represents the mean ± S.E. from three experiments, in which at least 30 cells were counted at each time point.

3.3. Geldanamycin causes formation of fluorescent globules

Treatment of NT2-N cells with both dexamethasone and geldanamycin resulted in the formation of fluorescent globules along the neurites. Fig. 4A is a view of a multinuclear gangliform structure with multiple neurites, each containing an alignment of fluorescent globules. These fluorescent globules can also be seen transiently in the cytoplasm of cells treated for short times (e.g., 10 min) with both dexamethasone and geldanamycin (see Fig. 1), but their presence in neurites is most striking. Fluorescent globules are not produced in cells treated only with dexamethasone or only with geldanamycin (Fig. 1). Thus, it seems that both steroid-induced GFP–GR movement and geldanamycin inhibition of dynamic GFP–GR·hsp90 heterocomplex assembly are required for formation of fluorescent globules. As shown in Fig. 4B, the globules were formed in a time-dependent manner, and by 60 min, about 75% of the neurites with fluorescence contained fluorescent globules.

3.4. Reversal of the geldanamycin blockade

Geldanamycin is a reversible inhibitor of hsp90, and washing of geldanamycin-treated 3T3 mouse fibroblasts reverses inhibition of GFP–GR movement from the cytoplasm to the nucleus [9]. In the experiment of Fig. 5, NT2-N cells were incubated for 60 min at 37 °C with dexamethasone and geldanamycin to permit formation of neurite globules as shown in Figs. 1 and 4A. The medium was then replaced with steroid-free medium with or without geldanamycin and the incubation was continued at 37 °C. It can be seen that the fluorescent globules disappear from the neurites more rapidly ($t1/2$ 40–60 min) in the absence of geldanamycin (Fig. 5, open circles) than when geldanamycin is present (Fig. 5, open squares). The same results are seen with or without the continued presence of steroid during the second incubation (data not shown).

The hsp90-based chaperone machinery protects hsp90 'client' proteins from ubiquitination and subsequent proteasomal degradation, and treatment of cells with geldanamycin increases the rate of turnover of hsp90-bound proteins [16], including the glucocorticoid receptor [6]. As shown by the solid squares in Fig. 5, the presence of the proteasome inhibitor MG-132 inhibited the loss of fluorescent globules in neurites of NT2-N cells maintained in the presence of geldanamycin. This suggests that the GFP–GR that is jammed up in globules is degraded when hsp90 function is inhibited. In contrast, the rate of loss of neurite globule

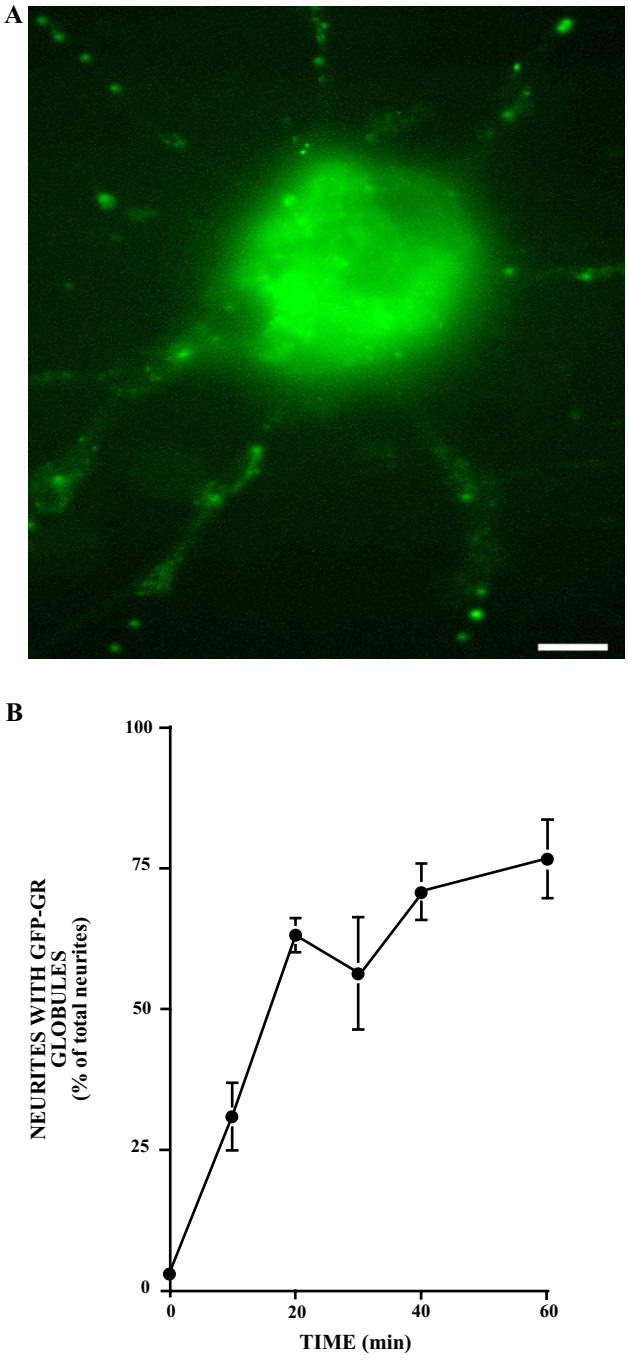

Fig. 4. Geldanamycin blockade of GR movement in axons and dendrites causes traffic jams that appear as fluorescent globules. (A) An enlarged view of a ganglion structure with multiple neurites that has been incubated for 60 min with dexamethasone and geldanamycin. The bar at the lower right represents 30 μm. (B) To determine GFP–GR in neurite globules, cells in Fig. 3 showing at least three fluorescent globules per neurite were scored as percent of the total number of cells exhibiting fluorescent neurites. Each value represents the mean ± S.E. from three experiments, in which at least 30 cells were counted at each time point.

fluorescence in NT2-N cells that were maintained in the absence of geldanamycin (Fig. 5, solid circles) was not affected by MG-132. This is consistent with the loss of neurite fluorescence upon removal of the geldanamycin block being due to GFP–GR exit from the globules and continued retrograde movement out of the neurites and to the nucleus.

3.5. CHIP and hsp70 co-localize in the GFP–GR fluorescent globules

The suggestion that the GFP–GR undergoes proteasomal degradation in the globules of neurites maintained in the presence of geldanamycin is buttressed by the observation that CHIP co-localizes to the globules. CHIP is a 35-kDa protein that binds via its TPR domain to the carboxyl terminus of both hsp70 and hsc70 [3]. CHIP possesses a carboxyl-terminal U-box that interacts with the ubiquitin-conjugating enzyme family [14]. CHIP has been shown to function as the E3 ubiquitin ligase for the GR to promote receptor ubiquitylation [4], thus initiating proteasomal degradation.

The localization of CHIP in NT2-N neurons is shown by the red fluorescence in Fig. 6. In untreated cells (not shown), cells treated with geldanamycin (Fig. 6a and b), and cells treated with dexamethasone (Fig. 6d), CHIP is located diffusely throughout the cytoplasm and the neurites. As shown in Fig. 1, the GFP–GR is diffusely present throughout the neurite in the absence of steroid (Fig. 6c) and moves to the nucleus with steroid treatment (Fig. 6e). In cells treated with both geldanamycin and dexamethasone, CHIP (Fig. 6g) and GFP–GR (Fig. 6h) co-localize (see merge in Fig. 6i) to the same globules, which are located periodically along the neurite.

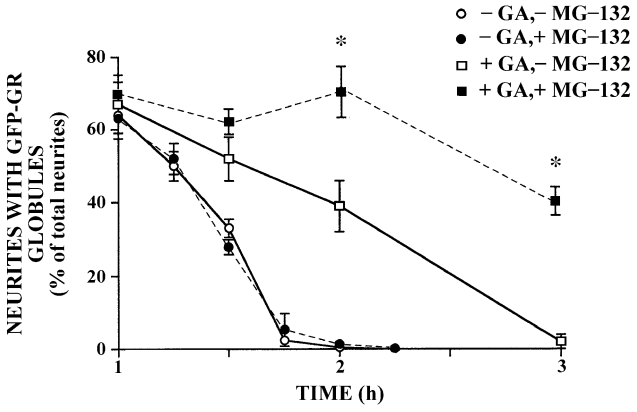

Fig. 5. Effects of geldanamycin withdrawal and treatment with the proteasome inhibitor MG-132. NT2-N cells expressing GFP–GR were incubated for 1 h in the presence of dexamethasone and geldanamycin to permit formation of fluorescent globules in the neurites. The medium was then replaced with steroid-free medium, and the incubation was continued at 37 °C under the following conditions: (○), without geldanamycin; (●) without geldanamycin but with 10 μM MG-132; (□) with 10 μM geldanamycin; (■) with geldanamycin and MG-132. At various times, the cells were fixed and neurites with GFP–GR globules were scored. Each value represents the mean ± S.E. from three experiments. Asterisks designate values for geldanamycin-treated cells that differ from the control without MG-132 at a significance of $P < 0.001$.

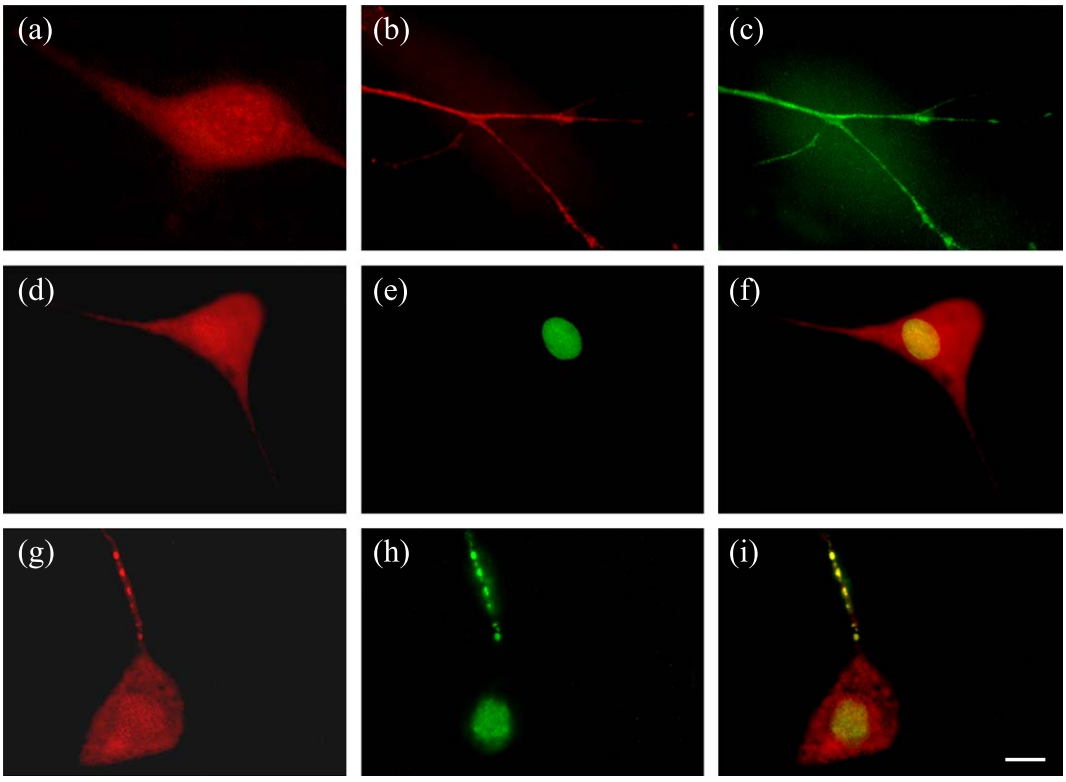

Fig. 6. CHIP co-localizes with the GFP–GR in fluorescent globules. NT2-N cells expressing GFP–GR were incubated for 1 h with geldanamycin (a–c), dexamethasone (d–f), or both geldanamcyin and dexamethasone (g–i). The red represents indirect immunofluorescence for CHIP and the green is GFP–GR. The distribution of CHIP in a cell body is shown in panel a, and panels b and c show the distribution of CHIP and GFP–GR, respectively, in a long neurite extending from that cell body. Panels f and i show the merged images of panels d–e and g–h, respectively. The bar at the lower right represents 10 μm.

In cells that are not overexpressing the GFP–GR, CHIP remains dispersed throughout the neurite, upon treatment with dexamethasone and gledanamycin, and there is no globule formation. Thus, the component of the proteasomal degradation system that triggers receptor ubiquitination, CHIP, appears to move with the receptor upon steroid

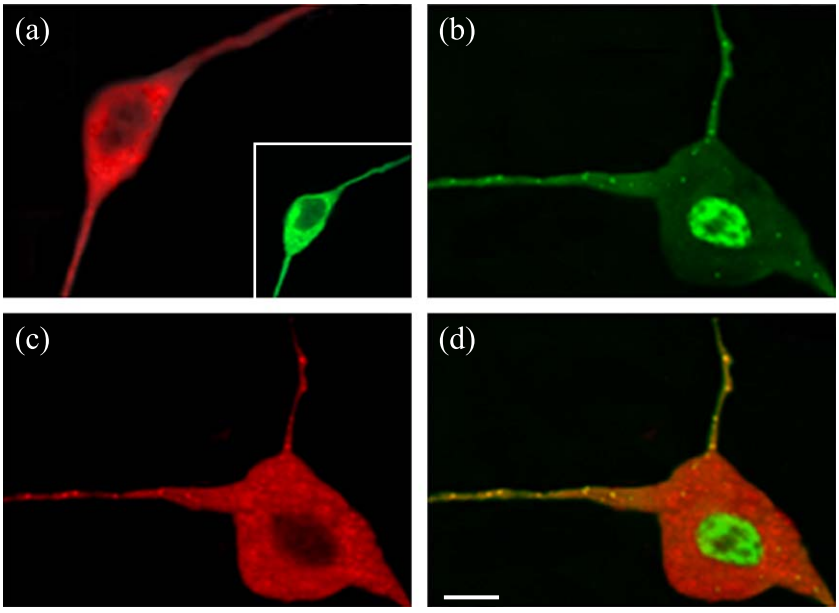

Fig. 7. Hsp70 co-localizes with the GFP–GR in fluorescent globules. NT2-N cells expressing GFP–GR were either untreated (a) or treated for 1 h with both geldanamycin and dexamethasone (b–d). The red represents indirect immunofluorescence for hsp70 and the green is GFP–GR. The inset in (a) shows the GFP–GR distribution in the same cell where the distribution of hsp70 is shown in red. Panel d shows the merged images of panels b and c. The bar in d represents 10 μm.

treatment and becomes jammed in the same loci when further receptor movement is blocked by geldanamycin inhibition of receptor·hsp90 heterocomplex assembly. We do not know the relative concentrations of CHIP and GFP–GR in the cell. However, as steroid-dependent transcriptional activation in GFP–GR transfected cultures rises more than 20-fold [8], the amount of GFP–GR produced in the ~5% of cells in the culture that are overexpressing it must be very high and may very well exceed that of CHIP. Such a change in the receptor/CHIP ratio would account for the receptor-dependent movement of CHIP to the fluorescent globules.

Because CHIP interacts via its TPR domain with GR-bound hsp70, it is reasonable to predict that some hsp70 would localize to the fluorescent globules. Despite the fact that hsp70 is an abundant protein, it can be shown that some of it is concentrated in the same fluorescent globules as the GFP–GR. Fig. 7a shows the distribution of hsp70 (red) in an untreated NT2-N neuron expressing GFP–GR (green in inset). It can be seen that hsp70 is distributed diffusely throughout the neurites. In transfected cells treated with dexamethasone and geldanamycin, GFP–GR (Fig. 7b) and some of the hsp70 (Fig. 7c) co-localize (see merge in Fig. 7d) to the same globules in the neurites. As with CHIP, hsp70 concentrates in neurite globules only in cells that are expressing GFP–GR that are treated with both geldanamycin and dexamethasone (data not shown). Similar indirect immunofluorescence localization experiments were performed for both hsp90 and the hsp90-binding immunophilin FKBP52. Both proteins could be readily visualized in neurites, but they did not localize to fluorescent granules in GFP–GR expressing cells treated with geldanamycin and dexamethasone (data not shown).

4. Discussion

There are several reasons why the GFP–GR is an excellent model for studying the mechanism of movement of a soluble protein in neurites. The presence of the fluorescent GFP does not affect the localization of the hormone-free receptor, and transcriptional activation by the chimera is similar to that of the wild-type GR [8]. In addition, the investigator can initiate GFP–GR movement from the neurite because movement is steroid-dependent. Importantly, a great deal is already known about the formation and composition of GR·hsp90 heterocomplexes and their linkage to a movement system in non-neuronal cells. These GR·hsp90 heterocomplexes immunoabsorbed from cytosols contain one of several hsp90-binding immunophilins and the motor protein, cytoplasmic dynein [7,10].

When assembly of this movement machinery is blocked by geldanamycin, rapid movement ceases, but receptors still accumulate slowly in the nucleus, both in non-neuronal cells [8–10] and in NT2-N neurons (Figs. 1 and 2). In fibroblasts, when the microtubular pathway for rapid motor-dependent movement is eliminated by colcemid treatment, both wild-type GR [5] and GFP–GR [8] move to the nucleus in a steroid-dependent manner. But movement in the absence of microtubules is not affected by geldanamycin, and thus, does not utilize the hsp90-dependent machinery [8]. In the absence of microtubules, GR that moves to the nucleus by diffusion is clearly functional because microtubules are not required for GR-mediated gene induction [24]. We should note here that colcemid treatment of NT2-N cells made the neurites disappear, so we could not ask whether microtubule disruption blocked GFP–GR movement in neurites. The rapid movement machinery is not essential for the GR to move to its nuclear site of action in yeast, because steroid-dependent transcriptional activation occurs in yeast lacking dynein [21].

Thus, in non-neuronal cells and in the cell body of NT2-N neurons (Fig. 2), the hsp90-dependent machinery is responsible for the rapid movement of the GR, but it is non-essential because the alternative of movement via diffusion is available. Clearly, movement systems for soluble proteins had to have evolved before cells could develop axons and dendrites where movement by diffusion is not possible and proteins must be moved by a machinery (probably multiple machineries). The observation that geldanamycin blocked GFP–GR movement in NT2-N cell neurites (Fig. 3) suggests that the hsp90-dependent movement machinery, which is non-essential for receptor movement in the cell body of neurons, is essential for receptor movement in axons and dendrites. Thus, in the integrated, multicellular organism possessing a nervous system, a function of hsp90 in protein trafficking that is non-essential in lower organisms may have become essential.

A particularly interesting observation is that geldanamycin treatment caused the GFP–GR to accumulate in fluorescent globules along the neurites (Figs. 1, 4, 6 and 7). GFP–GR movement must be initiated with steroid for the globules to form upon geldanamycin blockade of hsp90 function (Fig. 1). This has caused us to think of the globules as traffic jams. The GR must be in heterocomplex with hsp90 to bind steroid [19], and movement of the GFP–GR over a limited distance can occur before there is heterocomplex disassembly. This limited retrograde movement by steroid-bound GFP–GR that is complexed with hsp90 and linked via immunophilins to cytoplasmic dynein may account for the periodic localization of globules along neurites when further movement is stopped by geldanamycin blockade of heterocomplex reassembly.

Another possible explanation for the periodic location of the globules might be that the GFP–GR accrues in protein quality control centers containing the machinery for proteasomal degradation that are located periodically along the neurite. The proteasome inhibitor MG-132 inhibits the loss of fluorescent globules in the neurites of cells maintained in the presence of geldanamycin (Fig. 5), suggesting that the GFP–GR is being degraded at these sites. When the receptor is no longer protected from ubiquitination and

degradation because its assembly into heterocomplexes with hsp90 is blocked by geldanamycin, it might be degraded at such quality control loci along the neurite. It was with this in mind that we examined the localization of the CHIP and hsp70 components of the receptor degradation machinery in neurites. CHIP and hsp70 were dispersed throughout the neurites in untreated cells and in cells treated with only steroid or geldanamycin (Figs. 6 and 7 and data not shown). Thus, CHIP and hsp70 are not prelocated in protein quality control centers that are located periodically along the neurite. However, quality control centers for proteasomal degradation may exist, and CHIP and hsp70 may move with target proteins into such centers.

Quite unexpectedly, both CHIP and hsp70 were found to co-localize with the GFP–GR in the neurite globules when cells were treated with both steroid and geldanamycin (Figs. 6 and 7). CHIP and hsp70 movement to the globules occurred only in neurites expressing GFP–GR when movement of the receptor had been initiated with steroid. Thus, the CHIP and hsp70 moved to the globules with the receptor. CHIP and the GR have been shown to co-immunoprecipitate from cell lysates [4] where essentially all of the GR is in heterocomplex with hsp90. During GR·hsp90 heterocomplex assembly, the HOP component of the assembly machinery, which simultaneously binds hsp70 and hsp90 via independent TPR domains, dissociates [19]. This opens the TPR acceptor site on receptor-bound hsp70 to bind CHIP. Thus, CHIP may be able to dynamically interact with hsp70 bound to the GFP–GR while the hsp90 component of the heterocomplex determines retrograde movement by linking the complex to dynein via the TPR domain immunophilins. Thus, movement could occur while the E3 ubiquitin ligase interacts with the receptor complex. When continued GFP·GR·hsp90 heterocomplex assembly is blocked by geldanamycin, CHIP that has accompanied the receptor to the globules may function unopposed by the presence of hsp90, initiating receptor degradation by the proteasome. The fact that the GFP–GR that is jammed in globules is stabilized by the proteosome inhibitor MG-132 (Fig. 5) suggests that the globules function as degradasomes.

CHIP does not exist solely to regulate the GR, and it would seem at first glance that treatment with geldanamycin in the absence of dexamethasone might cause CHIP to aggregate in neurite globules because hsp90 chaperoning of other proteins that move to the nucleus is blocked. However, geldanamycin alone does not cause CHIP to localize to neurite globules (Fig. 6). This is probably because most nuclear proteins chaperoned by hsp90 move to the nucleus constitutively immediately upon completion of their translation and are never located in neurites. Because the GR shuttles in both directions with the great majority being located in the cytoplasm in the absence of hormone, a significant amount of GFP–GR is visualized in neurites. The extremely high levels of GFP–GR produced in the ~ 5% of overexpressing cells permit us to see CHIP accumulate in neurite globules when retrograde movement of the receptor is initiated by dexamethasone and hsp90 is blocked by geldanamycin. Regardless of the ultimate mechanistic explanation, it seems clear that both the CHIP and hsp70 components of the proteasomal degradation machinery must move with the receptor in the neurite, and the movement of both the receptor and the receptor-bound hsp70 and CHIP is blocked when hsp90 function is blocked by geldanamycin.

Acknowledgements

This work was supported by National Institutes of Health Grants CA28010 (to W.B.P.), NS23831 (to S.K.F.), DK47951 (to P.R.H.), and HL65619 and GM61728 (to C.P.). Cell Biology Core Laboratory services were supported in part by the Michigan Diabetes Research Training Center Grant P60DK-20572 from the NIDDK of the National Institutes of Health.

References

[1] R.T. Ambron, R. Schmied, C.C. Huang, M. Smedman, A signal sequence mediates the retrograde transport of proteins from axon periphery to the cell body and then to the nucleus, J. Neurosci. 12 (1992) 2813–2818.

[2] P.W. Andrews, Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro, Dev. Biol. 103 (1984) 285–293.

[3] C.A. Ballinger, P. Connell, Y. Wu, Z. Hu, L.J. Thompson, L.Y. Yin, C. Patterson, Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions, Mol. Cell. Biol. 19 (1999) 4535–4545.

[4] P. Connell, C.A. Ballinger, J. Jiang, Y. Wu, L.J. Thompson, J. Hohfeld, C. Patterson, The co-chaperone CHIP regulates protein triage decisions mediated by heat shock proteins, Nat. Cell Biol. 3 (2001) 93–96.

[5] M.J. Czar, R.H. Lyons, M.J. Welsh, J.M. Renoir, W.B. Pratt, Evidence that the FK506-binding immunophilin heat shock protein 56 is required for trafficking of the glucocorticoid receptor from the cytoplasm to the nucleus, Mol. Endocrinol. 9 (1995) 1549–1560.

[6] M.J. Czar, M.D. Galigniana, A.M. Silverstein, W.B. Pratt, Geldanamycin, a heat shock protein 90-binding benzoquinone ansamycin, inhibits steroid-dependent translocation of the glucocorticoid receptor from the cytoplasm to the nucleus, Biochemistry 36 (1997) 7776–7785.

[7] T.H. Davies, Y.M. Ning, E.R. Sanchez, A new first step in activation of steroid receptors, J. Biol. Chem. 277 (2002) 4597–4600.

[8] M.D. Galigniana, J.L. Scruggs, J. Herrington, M.J. Welsh, C. Carter-Su, P.R. Housley, W.B. Pratt, Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton, Mol. Endocrinol. 12 (1998) 1903–1913.

[9] M.D. Galigniana, P.R. Housley, D.B. DeFranco, W.B. Pratt, Inhibition of glucocorticoid receptor nucleocytoplasmic shuttling by okadaic acid requires intact cytoskeleton, J. Biol. Chem. 274 (1999) 16222–16227.

[10] M.D. Galigniana, C. Radanyi, J.M. Renoir, P.R. Housley, W.B. Pratt, Evidence that the peptidylprolyl isomerase domain of the hsp90-

binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus, J. Biol. Chem. 276 (2001) 14884–14889.
[11] M.D. Galigniana, J.M. Harrell, P.J.M. Murphy, M. Chinkers, C. Radanyi, J.M. Renoir, M. Zhang, W.B. Pratt, Binding of hsp90-associated immunophilins to cytoplasmic dynein: direct binding and in vivo evidence that the peptidylprolyl isomerase domain is a dynein interaction domain, Biochemistry 41 (2002) 13602–13610.
[12] N. Hirokawa, Kinesin and dynein superfamily proteins and the mechanisms of organelle transport, Science 279 (1998) 519–526.
[13] J. Hohfeld, D. Cyr, C. Patterson, From the cradle to the grave: molecular chaperones that may choose between folding and degradation, EMBO Rep. 2 (2001) 885–890.
[14] J. Jiang, C.A. Ballinger, Y. Wu, Q. Dai, D.M. Cyr, J. Hohfeld, C. Patterson, CHIP is a U-box-dependent E3 ubiquitin ligase. Identification of hsc70 as a target for ubiquitylation, J. Biol. Chem. 276 (2001) 42938–42944.
[15] M. Martin, S.J. Iyadurai, A. Gassman, J.G. Gindhart, T.S. Hays, W.M. Saxton, Cytoplasmic dynein, the dynactin complex, and kinesin are interdependent and essential for fast axonal transport, Mol. Biol. Cell 10 (1999) 3717–3728.
[16] L. Neckers, T.W. Shulte, E. Mimnaugh, Geldanamycin as a potential anti-cancer agent: its molecular target and biochemical activity, Invest. New Drugs 17 (1999) 361–373.
[17] J.E. Novak, R.S. Turner, B.W. Agranoff, S.K. Fisher, Differentiated human NT2-N neurons process a high intracellular content of *myo*-inositol, J. Neurochem. 72 (1999) 1431–1440.
[18] S.J. Pleasure, C. Page, V.M. Lee, Pure, postmitotic, polarized human neurons derived from Ntera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons, J. Neurosci. 12 (1992) 1802–1815.
[19] W.B. Pratt, D.O. Toft, Regulation of signal protein function and trafficking by the hsp90/hsp70-based chaperone machinery, Exp. Biol. Med. 228 (2003) 111–133.
[20] W.B. Pratt, A.M. Silverstein, M.D. Galigniana, A model for the cytoplasmic trafficking of signaling proteins involving the hsp90-binding immunophilins and $p50^{cdc37}$, Cell. Signal. 11 (1999) 839–851.
[21] D.L. Riggs, P.J. Roberts, S.C. Chirillo, J. Cheung-Flynn, V. Prapapanich, T. Ratajczak, R. Gaber, D. Picard, D.F. Smith, The hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling in vivo, EMBO J. 22 (2003) 1158–1167.
[22] S.M. Roe, C. Prodromou, R. O'Brien, J.E. Ladbury, B.W. Piper, L.H. Pearl, Structural basis for inhibition of the hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin, J. Med. Chem. 42 (1999) 260–266.
[23] A.M. Silverstein, M.D. Galigniana, K.C. Kanelakis, C. Radanyi, J.M. Renoir, W.B. Pratt, Different regions of the immunophilin FKBP52 determine its association with the glucocorticoid receptor, hsp90 and cytoplasmic dynein, J. Biol. Chem. 274 (1999) 36980–36986.
[24] D. Szapary, T. Barber, N.K. Dwyer, E.J. Blanchette-Mackie, S.S. Simons, Microtubules are not required for glucocorticoid receptor-mediated gene induction, J. Steroid Biochem. Mol. Biol. 51 (1994) 143–148.
[25] R.B. Vallee, M.A. Gee, Make room for dynein, Trends Cell Biol. 8 (1998) 490–494.
[26] C. Wang, D.J. Asai, K.R. Robinson, Retrograde but not anterograde bead movement in intact axons requires dynein, J. Neurobiol. 27 (1995) 216–226.
[27] C.M. Waterman-Storer, S.B. Karki, S.A. Kuznetsov, J.S. Tabb, D.G. Weiss, G.M. Langford, E.L.F. Holzbauer, The interaction between cytoplasmic dynein and dynactin is required for fast axonal transport, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 12180–12185.