Androgen Receptor Acetylation Site Mutations Cause Trafficking Defects, Misfolding, and Aggregation Similar to Expanded Glutamine Tracts*

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Kennedy's disease is a degenerative disorder of motor neurons caused by the expansion of a glutamine tract near the amino terminus of the androgen receptor (AR). Ligand binding to the receptor is associated with several post-translational modifications, but it is poorly understood whether these affect the toxicity of the mutant protein. Our studies now demonstrate that mutation of lysine residues in wild-type AR that are normally acetylated in a ligand-dependent manner mimics the effects of the expanded glutamine tract on receptor trafficking, misfolding, and aggregation. Mutation of lysines 630 or 632 and 633 to alanine markedly delays ligand-dependent nuclear translocation. The K632A/K633A mutant also undergoes ligand-dependent misfolding and aggregation similar to the expanded glutamine tract AR. This acetylation site mutant exhibits ligand-dependent 1C2 immunoreactivity, forms aggregates that co-localize with Hsp40, Hsp70, and the ubiquitin-protein isopeptide ligase (E3) ubiquitin ligase carboxyl terminus of Hsc70-interacting protein (CHIP), and inhibits proteasome function. Ligand-dependent nuclear translocation of the wild-type receptor and misfolding and aggregation of the K632A/K633A mutant are blocked by radicicol, an Hsp90 inhibitor. These data identify a novel role for the acetylation site as a regulator of androgen receptor subcellular distribution and folding and indicate that ligand-dependent aggregation is dependent upon intact Hsp90 function.

The androgen receptor (AR)1 is a ligand-activated transcription factor that is a pivotal regulator of sexually dimorphic traits. The receptor also plays a causative role in several human diseases including Kennedy's disease, a degenerative disorder of motor neurons caused by the expansion of a glutamine tract near the amino terminus of the receptor (1). Ligand-dependent translocation of the receptor to the nucleus is required both for normal AR function as a regulator of gene expression and for the neuronal pathology characteristic of Kennedy's disease (2, 3).

Kennedy's disease is one of a group of inherited neurodegenerative disorders caused by the expansion of CAG/glutamine tracts in affected genes (4). In all these disorders, the mutant proteins misfold and aggregate within the cytoplasm or nucleus. The presence of an expanded glutamine tract is the defining feature of these disorders and is causally related to the toxic gain-of-function conferred by the mutation. However, important differences among the polyglutamine expansion diseases exist, including the fact that distinct neuronal populations degenerate despite widespread expression of the mutant proteins. Such differences may be a consequence of altered normal function of the disease-causing protein. In Kennedy's disease, for example, the mutation causes a partial loss of AR function that may contribute to the specificity of the disease phenotype by depriving motor neurons of trophic support (5). Recent data also indicate that sequences outside the glutamine repeat may modulate toxicity of the mutant protein and contribute to selective neuronal vulnerability. In SCA1, another polyglutamine neurodegenerative disease, a phosphorylation site near the carboxyl terminus of ataxin-1 affects aggregation and toxicity of the mutant protein (6, 7). The identification of similar sequences that influence misfolding and toxicity of other disease-causing proteins could provide further insight into the pathogenic mechanisms underlying these diseases.

Among the CAG repeat disorders, the mutant protein with one of the best understood normal functions is AR. Much progress has been made in defining ligand-dependent post-translational modifications of the receptor that are required for regulated gene expression; however, far less is known about whether these modifications impact the toxicity of the mutant protein. Ligand binding to the receptor is accompanied by alterations in the tertiary structure of the receptor and by several post-translational modifications. Among these ligand-dependent modifications is the acetylation of lysine residues 630, 632, and 633 (5, 8). This acetylation may be mediated by the transcriptional co-activators p300/CREB-associated factor and p300 and was shown previously to affect ligand-regulated activation of hormone-responsive promoters. These lysine residues that are modified by acetylation are adjacent to the bipartite nuclear localization sequence of the receptor, which is encoded by amino acids 608–625 (9), and are in a region shown previously to affect nuclear trafficking (10). Since the expanded glutamine tract is known to delay the ligand-dependent nuclear translocation of the receptor (11) and to inhibit the activity of acetyltransferases (12–14), we sought to determine the effect of acetylation site mutations on AR. We now demonstrate that these lysine residues play a critical role in the regulation of the
subcellular localization and folding of the receptor. Mutation of these amino acids impairs ligand-dependent cytoplasmic to nuclear translocation and can lead to protein misfolding and aggregation similar to that which occurs in Kennedy's disease.

EXPERIMENTAL PROCEDURES

Materials—MN-1 cells and plasmids encoding Q24 or Q112 AR were from Dr. K. Fischbeck. K630A and K632A/K633A mutants (numbering based on a Q29 AR) were described previously (8), and GFPu was from Dr. R. Kopito. Antibodies against AR (N-20), Hsp90, and β-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Hsp40 was from StressGen (Victoria, British Columbia, Canada), and anti-Hsp70 was from Upstate Biotechnology (Lake Placid, NY). CHIP antibody, a gift from Dr. C. Patterson, was described previously (15). Anti-polyglutamine antibody (1C2) was from Chemicon (Tamecula, CA). FITC and Texas red-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). R1881 was from PerkinElmer Life Sciences. Hydroxyflutamide was from Schering-Plough.

Cell Transfection—HeLa cells were grown in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% charcoal and dextran-stripped fetal bovine serum. Cells were transfected with FuGENE 6 transfection reagent using 3 μl of FuGENE 6 and 1 μg of DNA. MN-1 cells stably expressing AR were isolated after co-transfection with pTracer EF/Bsd (Invitrogen) and indicated AR expression constructs. Cells were selected in medium supplemented with 7 μg/ml blasticidin. Clonal lines expressing equivalent levels of AR protein by Western blot were chosen for further analysis.

Western Blot Analysis—Cells were harvested, washed with phosphate-buffered saline, and lysed in radioimmune precipitation buffer. Protein samples were electrophoresed through a 10% SDS-polyacrylamide gel and transferred to Immunobilon-P membranes using a semi-dry transfer apparatus. Immunoreactive proteins were detected by chemiluminescence.

Quantification of AR Localization and Aggregation—Following fixation and staining, cells were visualized using a Zeiss Axiosplan 2 imaging system. AR localization was scored as described previously (16) using the following scale: 4 = nuclear fluorescence much greater than cytoplasmic fluorescence, 3 = nuclear fluorescence greater than cytoplasmic fluorescence, 2 = nuclear fluorescence equal to cytoplasmic fluorescence, 1 = nuclear fluorescence less than cytoplasmic fluorescence, and 0 = nuclear fluorescence much less than cytoplasmic fluorescence. AR aggregation was scored by determining the percentage of transfected cells with visible protein aggregates. For each experiment and each condition, >100 transfected cells were scored in a blind manner.

Immunofluorescence Microscopy—Cells cultured on chambered slides were fixed at −20 °C with methanol and stained. Confocal images were captured using a Zeiss LSM 510 microscope and a ×63 water immersion objective. For quantification, cells were examined using a Zeiss Axiosplan 2 imaging system.

Electron Microscopy—Cells were fixed in suspension with 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, overnight at 4 °C, and then post-fixed in 2% osmium tetroxide. After dehydration with ethanol and propylene oxide, cells were embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and observed on a Philips 400T transmission electron microscope.

Flow Cytometry—Cells were harvested by trypsinization, washed, and resuspended in phosphate-buffered saline. Cytometric analysis was performed using a Coulter Epics XL flow cytometer. Data analysis was performed using Winlist for Win 32 software. The cell population was gated to exclude necrotic cells and cell debris. 20,000 events within the gated region were analyzed per sample. Specific fluorescence of GFPu-expressing cells was determined by subtracting the background fluorescence of mock-transfected cells.

RESULTS

We first observed that point mutations in AR at lysine residues normally acetylated in a ligand-dependent manner disrupted the nuclear translocation of the receptor (Fig. 1). These initial studies utilized AR containing either 19 or 24 glutamines, both of which fall within the normal range of glutamine tract length in humans. Mutation of lysine residues 630 and 633 to alanine (K632A/K633A) shifted the distribution of the receptor toward the cytoplasm in the absence of ligand and delayed nuclear translocation of the receptor upon addition of ligand (Fig. 1A). Altered ligand-dependent translocation was observed in cells both transiently and stably expressing the mutant receptor. Clonal lines stably expressing wild-type or K632A/K633A mutant AR were established using MN-1 motor neuron-neuroblastoma hybrid cells. Treatment with ligand for 24 h revealed near complete nuclear translocation of the wild-type but not the mutant receptor (Fig. 1B). Similarly, lysine to alanine substitution of residue 630 (K630A) completely blocked
**FIG. 2.** The K632A/K633A mutant undergoes ligand-dependent aggregation. In A, HeLa cells expressing the Q24 or Q112 AR, with or without the K632A/K633A mutation, were treated with 10 nM R1881 or vehicle control for 24 h. AR localization was determined by indirect immunofluorescence using a FITC-conjugated secondary antibody. In B, cells expressing the Q24 AR with (closed bars) or without (open bars) the K632A/K633A mutation were treated with 10 nM R1881 for the indicated times, and AR was visualized by indirect immunofluorescence. The percentage of transfected cells containing aggregates was determined at each time point. Data are means ± S.E. of three independent experiments. C, electron micrographs of cell expressing the Q24 K632A/K633A mutant AR following treatment with 10 nM R1881 for 24 h. Left panel, electron-dense cytoplasmic aggregates. Magnification is at ×4600. Right panel, high magnification of cytoplasmic aggregates reveals an absence of surrounding membranes. Magnification is at ×10,000. In D, cells transfected with the indicated expression plasmids or pcDNA (control C) were treated with 10 nM R1881 or vehicle alone for 24 h. Lysates were analyzed by Western blot with antibodies against AR (top panel), expanded glutamine tract (1C2, middle panel), and β tubulin (bottom panel). In E, cells expressing Q24 AR with or without the K632A/K633A mutation (right and left panels, respectively) were treated with 10 nM R1881 for 24 h. 1C2 reactivity was assessed by indirect immunofluorescence, and nuclei were stained with DAPI. In F, cells expressing Q24 K630A AR were treated with 10 nM R1881 for 24 h. Lysates were analyzed by Western blot with antibodies against AR (top), expanded glutamine tract (1C2, middle), and β tubulin (bottom).
ligand-dependent nuclear translocation of the receptor, even up to 24 h following the addition of ligand (Fig. 1C). Subcellular localization of the androgen receptor was determined by indirect immunofluorescence and scored in >100 transfected cells expressing the K630A mutant following treatment with or without ligand for 24 h. This analysis revealed translocation scores of 0.63 ± 0.15 for cells treated with 10 nM R1881 and 0.54 ± 0.1 for cells treated with vehicle alone. These data indicated that lysine residues that are modified by acetylation are necessary for normal ligand-dependent nuclear translocation of AR. Decreased ligand-dependent nuclear accumulation may significantly contribute to the previously reported loss of receptor function conferred by these mutations (8, 17).

The K632A/K633A mutant exhibited both delayed nuclear entry and ligand-dependent misfolding and aggregation (Fig. 2A). Aggregates first appeared as early as 40 min after ligand addition and formed in ~60% of transfected cells following ligand treatment for 24 h (Fig. 2B). As in cells expressing AR with an expanded tract of 112 glutamines (Q112), aggregates of the K632A/K633A mutant formed predominantly in the cytoplasm and were not contained within membrane-bound structures such as lysosomes (Fig. 2, A and C). Ligand-dependent aggregation of the K632A/K633A mutant surprisingly produced AR misfolding similar to that caused by the presence of an expanded glutamine tract. Both the K632A/K633A mutant with 24 glutamines (Q24) and AR with an expanded tract of 112 glutamines were detected by the antibody 1C2 (Fig. 2D) that recognizes an epitope formed by misfolded proteins with long glutamine tracts. 1C2 immunoreactivity of the monomeric K632A/K633A mutant was detected on Western blot after the addition of ligand (Fig. 2D, lane 5) and correlated with the appearance of 1C2 reactive protein aggregates by immunofluorescence (Fig. 2E). No 1C2 reactivity of the K632A/K633A mutant was detected in the absence of ligand by immunofluorescence (data not shown). In contrast, the K630A mutant that failed to enter the nucleus in response to ligand did not form ligand-dependent aggregates (Fig. 1C), nor did it exhibit 1C2 immunoreactivity on Western blot (Fig. 2F).

Data from cell culture and animal models of CAG repeat disorders indicate that proteins with expanded glutamine tracts co-aggregate with chaperones and inhibit proteasome function (4, 18). We next sought to determine whether the Q24 K632A/K633A mutant similarly co-localized with chaperones and affected proteasome function in a ligand-dependent manner. Chaperones Hsp40 and Hsp70 co-localized with aggregates formed by the mutant receptor (Fig. 3, a–f), as they do with aggregates formed by proteins with expanded glutamine tracts. The E3 ubiquitin ligase CHIP (19) and GFP targeted for degradation by the proteasome (GFPu) (20) also co-localized with aggregates formed by the K632A/K633A mutant (Fig. 3, g–l). In contrast, GFPu was diffusely distributed in the cytoplasm of ligand-treated cells co-transfected with wild-type Q24 AR (data not shown). Redistribution of Hsp90 to aggregates was not detected (Fig. 3, m–o).

To determine whether ligand-dependent aggregation of the K632A/K633A mutant inhibited proteasome function, HeLa cells were co-transfected with GFPu plus wild-type AR, the K632A/K633A mutant, or the Q112 receptor. Following treatment with or without ligand for 24 h, mean fluorescence was determined by flow cytometry (Fig. 4A). Ligand-dependent increase in mean fluorescence, reflecting ligand-dependent inhibition of proteasome function (20), was significantly greater in cells co-transfected with the K632A/K633A mutant than in cells co-transfected with the wild-type receptor. Q112 AR also significantly increased mean fluorescence in a ligand-dependent manner. In contrast, treatment with the nonsteroidal androgen antagonist hydroxyflutamide caused neither aggregation (data not shown) nor proteasome inhibition (Fig. 4B). Inhibition of proteasome function by the expanded glutamine AR may contribute to the development of ligand-dependent neurodegeneration in Kennedy’s disease.

Delayed nuclear entry and ligand-dependent aggregation were striking features of the K632A/K633A mutant, whereas marked inhibition of nuclear translocation characterized the K630A mutant. In both cases, these point mutations caused either partial or near complete disruption of the mechanism that normally mediates ligand-dependent nuclear translocation. This mechanism, which has been well characterized for the glucocorticoid receptor, is based upon Hsp90-mediated linking of the steroid hormone receptor to the dynein motor protein via interaction with immunophilins (21). A similar Hsp90-mediated mechanism regulates nuclear translocation of AR (22). To confirm that ligand-dependent nuclear translocation of wild-type AR was Hsp90-dependent, cells at 4°C were treated first with ligand to engage the receptor and then with the Hsp90 inhibitor radicicol. AR subcellular localization was determined following a 4-h incubation at 37°C (Fig. 5A). Treatment with radicicol significantly inhibited ligand-dependent nuclear entry of wild-type AR, resulting in a distribution near baseline. These data indicated that translocation was an Hsp90-dependent process. We next sought to determine whether ligand-dependent aggregation of the K632A/K633A mutant was dependent upon Hsp90. Cells expressing the mutant receptor were incubated at 4°C with ligand alone, ligand then radicicol, or ligand then radicicol plus the proteasome inhibitor lactacystin. AR aggregation was assessed after a 4-h incubation at 37°C (Fig. 5, B and C). Radicicol significantly decreased ligand-dependent misfolding and aggregation of the mutant receptor, as assessed both by direct counts...
of cells containing aggregates and by the appearance of 1C2 immunoreactive monomeric AR on Western blot. Although the induction of 1C2 immunoreactivity was less intense after a 4-h ligand treatment than after a 24-h treatment (compare Figs. 2D and 5C), the shorter induction period was chosen for these experiments to avoid toxicity associated with prolonged exposure to radicicol. The effect of radicicol on misfolding and aggregation was not due to enhanced AR degradation by the proteasome since a similar effect was observed in the presence of lactacystin. Taken together, these data indicated that ligand-dependent nuclear translocation of AR was dependent upon Hsp90. Mutation of lysine residues 632 and 633 partially inhibited this process and caused ligand-dependent misfolding and aggregation of the receptor that required intact Hsp90 function.

**DISCUSSION**

Recent studies have shown that AR undergoes ligand-dependent acetylation at lysine residues present in a conserved motif adjacent to the DNA-binding domain (8). Acetylation at these sites has been implicated to play an important role in AR activation by influencing interaction with transcriptional co-regulators (17). Our data now demonstrate that mutation of the acetylation site also impairs ligand-dependent trafficking of the receptor from the cytoplasm to the nucleus and may promote receptor misfolding and aggregation. These studies thus identify another mechanism by which mutation of the AR acetylation site decreases receptor function as a ligand-dependent transcriptional activator.

Ligand-dependent misfolding and aggregation of the K632A/K633A mutant is reminiscent of ligand-dependent aggregation of the expanded glutamine AR that causes motor neuron degeneration in Kennedy's disease. Both the K632A/K633A mutant and the Q112 receptor form ligand-dependent aggregates in cell culture that are predominantly cytoplasmic. The presence of cytoplasmic aggregates or microaggregates may contribute to the disruption of axonal transport by the expanded glutamine AR (23, 24). Both the misfolded K632A/K633A mutant and Q112 AR are recognized by the antibody 1C2, aggrec-
gate with molecular chaperones, and may inhibit proteasome function. Chaperones Hsp40 and Hsp70, the E3 ubiquitin ligase CHIP, and GFP targeted for degradation by the proteasome co-localize with aggregates formed by the K632A/K633A mutant, suggesting that these represent sites of protein degradation. In contrast, Hsp90 is required for ligand-dependent transport of AR but does not redistribute to the aggregates. Misfolding and aggregation of the K632A/K633A mutant are associated with diminished proteasome activity in cells co-transfected with GFPu. Although a similar approach was used previously to measure altered proteasome function in intact cells (20), it is also possible that other mechanisms might contribute to the observed increased fluorescence.

Decreased acetylation of histones and diminished activity of the acetyltransferase cAMP-response element-binding protein (CBP) occur in several models of CAG repeat disorders, including cellular and Drosophila models of Kennedy’s disease (12–14). Our data suggest that decreased acetylation of the expanded glutamine AR may contribute to its
propensity to form ligand-dependent protein aggregates by interfering with Hsp90-mediated cytoplasmic to nuclear transport. The inhibitory effect of radicicol on ligand-dependent misfolding and aggregation may appear surprising given the well-established role of Hsp90 in promoting correct protein folding and facilitating degradation of misfolded proteins. However, Hsp90 also plays a critical role in ligand-dependent movement of the androgen receptor and other steroid hormone receptors (21). Our data indicate that Hsp90-dependent movement is required for misfolding and aggregation of the K632A/K633A mutant receptor that occurs in the presence of ligand. Consistent with this interpretation is the observation that the K630A mutant does not exhibit ligand-dependent movement, nor does it show ligand-dependent misfolding and aggregation. Like misfolding and aggregation, delayed ligand-dependent nuclear entry is a feature shared by both the K632A/K633A acetylation site mutant and the expanded glutamine receptor (11). Such abnormal receptor trafficking may contribute to the partial loss of AR function that occurs in Kennedy’s disease. Recent findings suggest that sequences outside the glutamine tract may influence aggregation and toxicity of other proteins with long glutamine repeats. In this context, our data complement reports demonstrating the role of Akt-mediated phosphorylation of ataxin-1 as a modifier of aggregation and toxicity (6,7).

AR is subject to ligand-dependent acetylation likely mediated by the co-activators p300, p300/CREB-associated factor, or Tip60 (8, 25). It is currently unknown in which cellular compartment AR is acetylated and whether other, predominantly cytoplasmic, acetyltransferases may also mediate this process. Disruption of ligand-dependent AR acetylation in motor neurons may contribute to the neuronal vulnerability that characterizes Kennedy’s disease. None of the other proteins mutated in the polyglutamine expansion diseases are known to undergo regulated acetylation, suggesting that the influence of the acetylation site on folding and aggregation may be unique to AR. It will be interesting to define which factors mediate ligand-dependent acetylation of AR in motor neurons and to determine whether the activity of these factors is altered in Kennedy’s disease.

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