Role of the ubiquitin ligase E6AP/UBE3A in controlling levels of the synaptic protein Arc

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Inactivation of the ubiquitin ligase E6 associated protein (E6AP) encoded by the UBE3A gene has been associated with development of the Angelman syndrome. Recently, it was reported that in mice, loss of E6AP expression results in increased levels of the synaptic protein Arc and a concomitant impaired synaptic function, providing an explanation for some phenotypic features of Angelman syndrome patients. Accordingly, E6AP has been shown to negatively regulate activity-regulated cytoskeleton-associated protein (Arc) and it has been suggested that E6AP targets Arc for ubiquitination and degradation. In our study, we provide evidence that Arc is not a direct substrate for E6AP and binds only weakly to E6AP, if at all. Furthermore, we show that down-regulation of E6AP expression stimulates estradiol-induced transcription of the Arc gene. Thus, we propose that Arc protein levels are controlled by E6AP at the transcriptional rather than at the posttranslational level.

Posttranslational modification of proteins by ubiquitin and ubiquitin like (UBL) proteins plays a prominent role in the regulation of many eukaryotic processes (1, 2). In recent years, components of the respective conjugation systems have emerged as potential targets in the treatment of human diseases because their deregulation has been associated with the development of distinct disorders or because they control pathways that, for instance, are of fundamental importance for the proliferative potential of cancer cells (3, 4). An impressive example for the latter is represented by the E3 ubiquitin ligase E6 associated protein (E6AP), which is encoded by the UBE3A gene on chromosome 15q11.3 (5, 6) and has been linked to three distinct disorders. Firstly, E6AP was originally isolated as an interacting protein of the E6 protein of onco genic human papillomaviruses (HPVs) (7, 8). In complex with E6, E6AP targets proteins for degradation [e.g., the tumor suppressor protein p53] that are normally not recognized by E6AP, thereby contributing to HPV induced cervical carcinogenesis (9, 10). Secondly, loss of E6AP expression or function results in the development of Angelman syndrome (11, 12), a neurodevelopmental disorder (11–14). Finally, deregulation of E6AP expression has been associated with the development of distinct potential targets in the treatment of human diseases because their deregulation has been associated with the development of distinct disorders or because they control pathways that, for instance, are of fundamental importance for the proliferative potential of cancer cells (3, 4). An impressive example for the latter is represented by the E3 ubiquitin ligase E6 associated protein (E6AP), which is encoded by the UBE3A gene on chromosome 15q11.3 (5, 6) and has been linked to three distinct disorders. 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To determine the effect of E6AP on Arc stability, Arc was expressed as a DHFR ubiquitin fusion protein in the absence or presence of E6AP or a catalytically inactive E6AP mutant (E6AP C820A) in HEK293T cells in which endogenous E6AP expression is knocked down by RNA interference (Fig. 2B). As controls, Ring1b I53S (an inactive form of the E3 ligase Ring1b), which is a substrate for E6AP (23), and an N terminally truncated form of HECT domain and RCC1 like domain containing protein 2 (HERC2_tr) (39), which serves as an artificial substrate of E6AP (for further details, see the legend to Fig. 2C), were used. This revealed that coexpression of E6AP had no significant effect on Arc levels (Fig. 2C). In contrast, levels of Ring1b I53S and HERC2_tr were significantly reduced in the presence of E6AP but not in the presence of E6AP C820A (Fig. 2C). Similarly, coexpression of E6AP had no significant effect on the levels of ubiquitinated Arc, whereas it facilitated (mainly) monoubiquitination of Ring1b I53S (Fig. 2D). Thus, the results obtained indicate that within cells, Ring1b I53S and HERC2_tr, but not Arc, are targeted by E6AP for ubiquitination and degradation.

**Knockdown of E6AP Expression Stimulates Estradiol-Induced Transcription of the Arc Gene.** It was recently reported that transgenic mice carrying two additional Ube3a alleles show autism like features (17). The additional Ube3a alleles were engineered such that they express E6AP with a C terminal Flag tag. To provide evidence that “E6AP Flag” still has E3 activity, it was shown that in the transgenic mice, Arc levels are significantly decreased in brain derived lysates (17). However, it was previously shown that fusing a C terminal extension (e.g., Flag tag) to E6AP or deleting the six C terminal residues (E6AP ΔC6) result in ubiquitination defective proteins (40). Indeed, like E6AP ΔC6 and the E6AP C820A mutant, E6AP Flag as well as E6AP GFP (Flag tag or GFP fused to the C terminus of E6AP) did not target HERC2_tr for degradation within cells, whereas GFP E6AP (GFP fused to the N terminus of E6AP) was active (Fig. 2E). Similarly, E6AP Flag and E6AP GFP were not able to facilitate HPV E6 mediated degradation of p53 but rather acted as dominant negative mutants (Fig. 2F), supporting the notion that fusing a C terminal extension to E6AP impairs its E3 activity. Thus, the autism like features of Ube3a Flag transgenic
mice (17) do not appear to be caused by an increase in E3 activity but rather by inhibition of the E3 activity of endogenous E6AP and/or by an increase in E3 independent properties of E6AP. How can the data obtained so far be reconciled with the observations that mice overexpressing E6AP Flag have reduced Arc levels (17) and that under certain conditions, Arc levels are

Fig. 2. Arc does not represent a substrate for E6AP in cells. (A) Schematic of the DHFR HA ubiquitin fusion protein system. For details, see Results. (B) Extracts were prepared from HEK293T cells (par) and HEK293T cells, in which endogenous E6AP expression is stably down regulated by RNA interference (shE6AP), and subjected to Western blot analysis with antibodies against E6AP and tubulin (Tub). (C) HEK293T shE6AP cells were cotransfected with expression constructs for HA tagged E6AP or the inactive mutant E6AP C820A (HA C820A), and DHFR HA ubiquitin fusion proteins of HA tagged Ring1b I53S (R1b), HA tagged Arc, or HA tagged HERC2 tr. Protein extracts were prepared 24 h after transfection. Levels of the various proteins were determined by Western blot analysis using an anti HA antibody and quantified. The relative ratio of HA tagged Ring1b I53S, Arc, or HERC2 tr to DHFR HA ubiquitin in the absence of E6AP set to 100%. Running positions of DHFR HA ubiquitin are indicated by closed circles; those of HA tagged Ring1b I53S, Arc or HERC2 tr are indicated by asterisks, and those of E6AP and E6AP C820A are indicated by arrowheads. Running positions of molecular mass markers (kDa) are marked. Ring1b I53S was used as substrate, because it cannot ubiquitinate itself (23). Thus, its degradation by E6AP can be easily monitored. HERC2 tr is an N terminal truncation mutant of HERC2 (amino acids 2958 4834), and, unlike full length HERC2 (39), it is an efficient substrate for E6AP. (D) Transfections were performed as in C but in the presence of a construct encoding His tagged ubiquitin (His ub). Protein extracts were prepared and ubiquitinated proteins were isolated by Ni²⁺ affinity chromatography (Ni PD). Levels of ubiquitinated Ring1b I53S (R1b) and Arc were determined by Western blot analysis with antibodies against Ring1b or Arc. Running positions of molecular mass markers (kDa) are indicated. * indicates (presumably mono) ubiquitinated forms of Ring1b I53S and Arc. Input corresponds to 15% of the extracts used for affinity purification. Running positions of E6AP, Ring1b I53S, Arc, and DHFR ubiquitin are indicated. (E) H1299 cells, in which endogenous E6AP expression is stably down regulated (45), were cotransfected with constructs encoding HA tagged HERC2 tr, various forms of E6AP as indicated, and β galactosidase (to determine transfection efficiency). Twenty four hours after transfection, extracts were prepared and levels of HERC2 tr (Upper) and of the various forms of E6AP (Lower) were determined by Western blot analysis and quantified; mock, mock transfected cells; HA E6AP Flag, HA E6AP with a C terminal Flag tag; HA E6APΔC6, C terminally truncated E6AP (deletion of the C terminal 6 amino acids) with an N terminal HA tag; GFP E6AP, GFP fused to the N terminus of E6AP; E6AP GFP, GFP fused to the C terminus of E6AP. Running positions of the HA tagged forms and GFP fusions of E6AP are indicated by a closed circle or an arrowhead, respectively. Running positions of molecular mass markers (kDa) are indicated. (F) As in E, but parental H1299 cells were used for transfection and p53 was used as substrate for the E6AP HPV E6 complex. Note that endogenous E6AP is sufficient for E6 mediated degradation of p53 (45). Thus, the finding that coexpression of inactive forms of E6AP (HA E6APΔC6, HA C820A) and of C terminal fusions of E6AP (HA E6AP Flag, E6AP GFP) interferes with E6 mediated degradation of p53 indicates that these proteins are not only inactive but, moreover, act as dominant negative mutants. Experiments shown in C F were repeated at least three times with similar results.
increased in brain lysates derived from Ube3a null mice (26)? E6AP was reported to affect nuclear hormone receptor mediated transcription by E3 independent and E3 dependent mechanisms (41–43). Furthermore, expression of the human Arc gene is in ceased by estradiol signaling (44). We, therefore, hypothesized that loss of E6AP results in increased estrogen receptor (ER) mediated transcription and thereby Arc expression. To address this possibility, we first studied ER induced transcription of a respective reporter construct in H1299 shE6AP cells, in which E6AP expression is down regulated by RNA interference (45) and in parental H1299 cells. Indeed, ER induced expression of the reporter gene was approximately twofold higher in H1299 shE6AP cells than in H1299 cells (Fig. 3A). In contrast, ectopic expression of E6AP decreased ER induced transcription by a factor of two to three, and this repressive effect was not dependent on E3 activity (Fig. 3B).

Having shown that loss of E6AP expression stimulates ER induced transcription of a reporter gene, we then determined the effect of estradiol treatment on endogenous Arc mRNA levels in neuroblastoma derived SH SY5Y cells and in SH SY5Y shE6AP cells, in which E6AP expression is down regulated by RNA interference (Fig. 4A). As reported (44), treatment of parental SH SY5Y cells with estradiol resulted in a transient increase of Arc mRNA levels (Fig. 4B). In line with the results obtained with the ER responsive reporter construct, this increase was 2.3 times higher in SH SY5Y shE6AP cells (Fig. 4B and C). In conclusion, our data indicate that at least under certain conditions, E6AP constrains estradiol induced transcription of the Arc gene.

Discussion

All of the known genetic abnormalities associated with AS development result in loss of E6AP expression or expression of E6AP mutants with reduced E3 activity (11, 12, 46, 47), indicating that constitutive or transient increases in the level of substrate proteins of E6AP play a major role in AS development. Arc is a crucial player in synaptic plasticity, whose deregulation contributes to the manifestation of AS (24, 48, 49). Thus, the finding that under certain conditions, Arc levels are elevated in E6AP null mice (26) likely explains some of the symptoms of AS patients. Although our data support the notion that loss of E6AP expression results in regulation of Arc, we found that E6AP negatively affects Arc expression at the transcriptional rather than at the posttranslational level, as proposed previously (26).

In contrast to a previous report (26), we were not able to obtain evidence that E6AP binds to Arc with rather low affinity and, thus, detection of this interaction depends on the conditions used. The finding that E6AP ubiquitates Arc in vitro (Fig. S2) (26) may support this possibility. However, as indicated above (also see SI Results and Discussion), we propose that E6AP mediated ubiquitination of Arc in vitro is a non specific process. In other words, in vitro ubiquitination studies are important to support the notion that a protein is a substrate for E6AP; however, additional criteria need to be met to conclude that the ubiquitination observed in vitro is physiologically relevant.

An important cornerstone in the line of arguments that a certain E3 ligase regulates the stability of a given protein is the verification that the E3 ligase affects the ubiquitination status and the turnover rate of the respective protein in cells in coexpression experiments. Similar to our data (Fig. 2D), it was reported that ubiquitination of Arc is observed in the absence of ectopically expressed E6AP (26). In contrast to our data, however, coexpression of E6AP and Arc resulted in somewhat increased levels of ubiquitinated Arc, whereas ubiquitinated Arc species were not detected in the presence of an
inactive E6AP mutant. A potential drawback of cotransfection experiments is that transfection efficiencies can vary between in
dividual transfections and, thus, transfection efficiencies need to be considered when interpreting results obtained in such experiments.
To do so, the DHFR ubiquitin fusion protein system (36, 38, 45) is ideally suited, because the relative transfection efficiency can be
duly determined by monitoring the level of DHFR ubiquitin (Fig. 2A). Using this system, we showed that E6AP has neither a significant effect on the ubiquitination status nor on the level of
ectopically expressed Arc (Fig. 2C and D). The latter result is again in contrast to published data (26). However, it should be noted that in the respective study, data from proteasome inhibitor or half life
experiments to indicate that the decrease in Arc levels is indeed attributable to enhanced degradation rather than to effects on, for
instance, the transcriptional or translational level were not provided (26). Such experiments are not required when using the DHFR
ubiquitin fusion protein system, because DHFR ubiquitin and the POI are expressed as a single polypeptide from the same mRNA.
Thus, determination of the relative ratio between the POI and DHFR ubiquitin provides unequivocal evidence as to whether a
protein is preferentially degraded or not.
In support of the notion that Arc is an E6AP substrate, it was reported that upon treatment of hippocampal cells with kainate, Arc
protein levels but not Arc mRNA levels are increased in E6AP null cells (26). However, it is known that in response to various stimuli, Arc transcription is induced but only transiently (e.g., refs. 44, 50, and 51). For example, similar to our results (Fig.
4), it was reported that treatment of SH SY5Y cells with estradiol or carbachol results in a rapid but transient induction of Arc
mRNA levels and that this transient increase in mRNA levels is mirrored in a transient increase in Arc protein levels but with
different kinetics (44, 50). Because in the study with kainate (26), the analysis was performed at a single time point (and not in
a time course experiment), we propose that the increase of Arc protein levels observed in E6AP null cells was attributable to a
transient increase in mRNA levels rather than a prolonged half life of the Arc protein.
Besides challenging the previously proposed mechanism by which E6AP regulates Arc levels (26), our data suggest that the
importance of the connection between E6AP and nuclear hor
mone receptors for development of AS (and, possibly, autism
spectrum disorders; Results) has been underestimated so far. Estrogen was reported to affect Arc transcription (44), as well as
various neuronal processes including synaptic plasticity (52–56).
Furthermore, >5 10% of AS patients do not harbor a detectable
defect in the UBE3A gene (12). Thus, although we do not yet
know how E6AP interferes with estradiol induced transcription of the Arc gene [E6AP can affect transcriptional processes by
E3 dependent and E3 independent mechanisms (41-43)], it is
tempering to speculate that some AS patients carry defects along the
respective signaling pathway(s). Furthermore, analysis of
transgenic mice, in which E6AP expression is ablated in mammary
tissue, identified a few genes, the estradiol induced expression of
which is down regulated in the absence of E6AP (57). Similarly,
preliminary data (Fig. S3) suggest that in contrast to the Arc gene,
down regulation of E6AP expression in SH SY5Y cells results in
a decrease in estradiol induced transcription of the cathepsin D
gene, an established estradiol responsive gene (58, 59). Taken
together, these data support the notion that E6AP has both posi
tive and negative effects on estradiol mediated transcription.
Thus, delineation of the mechanism(s) involved in this phenon
enon and identification of the genes whose expression is altered in
the absence of E6AP should contribute to the elucidation of the
pathways involved in AS development.

Materials and Methods

For cell lines, plasmids, transfection procedures, antibodies, coprecipitation, and luciferase reporter assays, see SI Materials and Methods.

Ubiquitination and Degradation Assays. For ubiquitination assays, one 6 cm plate of cells was transfected with expression constructs encoding E6AP or
E6AP C820A (2.5 μg), His tagged ubiquitin (1.5 μg), and DHFR HA ubiquitin
HA Ring1B I535 or DHFR HA ubiquitin HA Arc (1 μg). Twenty four hours
after transfection, 30% of the cells were lysed under non denaturing condi
tions (30) to determine levels of E6AP, E6AP C820A, DHFR HA ubiquitin,
HA Ring1B I535, and HA Arc. The remaining cells were lysed under denaturing
conditions and ubiquitinated proteins were purified as described (30).

Degradation assays with DHFR ubiquitin fusion proteins were performed
similar to ubiquitination assays, except that 3.5 and 1 μg of the constructs
encoding E6AP and E6AP C820A, respectively, were used and the construct
for His ubiquitin was omitted. Quantification of the intensity of the signals
was performed with the Aida 4.0B software package (Raytest). HPV E6
induced degradation of p53 was monitored as described (45).

Estradiol Treatment and Real Time PCR. Cells were starved overnight in phenol
red free medium containing 5% (vol/vol) charcoal stripped FBS. Medium was
exchanged and cells were treated with 10 nM 17β estradiol (Sigma). Ap
proximately 5× 105 cells were harvested at the times indicated (Fig. 4), and
RNA was isolated by using TRizol Reagent (Invitrogen) and a Genejet RNA
puriﬁcation column (Fermentas). Reverse transcription was performed using
the SuperScript III Reverse Transcriptase Kit (Invitrogen). The resulting cDNA
was puriﬁed using the GeneJet PCR puriﬁcation kit (Fermentas). Quantitative
PCR analysis was performed using the Maxima SYBR Green/ROX qPCR Master
Mix (Fermentas) or FastStart Universal SYBR Green Master Mix (ROX) (Roche).

mRNA levels were compared using the ΔΔ Ct method (60, 61). Actin mRNA
was chosen as reference target. Primers used were as follows: Arc forward,
CCGGGATGGTTCTAC, Arc reverse, AGCCAGTACTCCTCAG, Actin forward,
GGTCGGGATGTCGA; and Actin reverse, TGGCACCACACCTTCTAC.

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