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The Glutamine-Alanine Repeat Domain of TCERG1 Is Required for the Inhibition of the Growth Arrest Activity of C/EBPα

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ABSTRACT

TCERG1 was characterized previously as a repressor of the transcription factor C/EBPα through a mechanism that involved relocalization of TCERG1 from nuclear speckles to pericentromeric regions. The inhibitory activity as well as the relocalization activity has been demonstrated to lie in the amino terminal half of the protein, which contains several discrete motifs including an imperfect glutamine-alanine (QA) repeat. In the present study, we showed that deletion of this domain completely abrogated the ability of TCERG1 to inhibit the growth arrest activity of C/EBPα. Moreover, the QA repeat deletion mutant of TCERG1 lost the ability to be relocalized from nuclear speckles to pericentromeric regions, and caused an increase in the average size of individual speckles. We also showed that deletion of the QA repeat abrogated the complex formation between TCERG1 and C/EBPα. Examination of mutants with varying numbers of QA repeats indicated that a minimal number of repeats are required for inhibitory activity as well as relocalization ability. These data contribute to our overall understanding of how TCERG1 can have gene-specific effects in addition to its more general roles in coordinating transcription elongation and splicing. J. Cell. Biochem. 117: 612–620, 2016.

KEY WORDS: TCERG1; CCAAT ENHANCER BINDING PROTEIN; NUCLEAR SPECKLES; CONFOCAL MICROSCOPY; GLUTAMINE-ALANINE REPEAT; SUBNUCLEAR COMPARTMENTALIZATION

Transcriptional Elongation Regulator 1 (TCERG1) has been most studied in the context of regulating the rate of transcription by RNA polymerase II and the roles that it plays in alternative splicing. Proteomics analysis has shown that it interacts with RNA polymerase II [Carty et al., 2000; Carty and Greenleaf, 2002; Liu et al., 2013] and several splicing factors such as SF1 [Goldstrohm et al., 2001]. These multiple protein interactions are possible due to the diverse array of functional motifs in TCERG1 that include several WW and FF domains through which it interacts with its protein partners [Lin et al., 2004; Smith et al., 2004; Sanchez-Alvarez et al., 2006).

The interaction of TCERG1 with RNA polymerase II and splicing factors suggests that its effects on the cellular genome would be broad. In support of this, Pearson et al. [2008] showed that the expression of over 900 genes was impacted by siRNA-mediated knockdown of TCERG1 expression. However, there is a body of evidence that supports the hypothesis that TCERG1 does have gene-specific effects which it exerts through different mechanisms. One of the first published studies on TCERG1 (initially called CA150) by Sune et al. highlighted this gene specificity by showing that only a subset of promoters were inhibited by TCERG1 over-expression [Sune and Garcia-Blanco, 1999].

Studies by our group and others have suggested some mechanisms whereby gene-specific effects of TCERG1 can be achieved. In experiments analyzing the mechanism of action of DACH1, a cell fate determination transcription factor, Zhou et al. [2010] identified TCERG1 as a specific interactor using a proteomics approach. Using reporter gene assays, they were able to characterize TCERG1 as a co-repressor for DACH1. One of the six FF domains present in the carboxy terminus of TCERG1, FF2, was required for binding to DACH1 and for its co-repressor activity.

Work from our lab identified yet another mechanism whereby TCERG1 can target specific genes. As part of our attempts to identify interactors of the transcription factor C/EBPα, we performed a yeast

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two-hybrid screen using a human liver cDNA library and pulled out TCERG1 as a potential interactor [McFie et al., 2006]. Our initial characterization suggested that TCERG1 was a co-repressor of C/EBPα, based on the observations that (i) the two proteins could be co-immunoprecipitated from mouse liver nuclear extracts and (ii) that over-expression of TCERG1 was able to inhibit C/EBPα-dependent promoter activation. However, the co-repressor label was re-evaluated after we examined the effect of TCERG1 on the HNF6 promoter, which is a promoter that is inhibited by C/EBPα [Banman et al., 2010]. While it was expected that TCERG1 would enhance the C/EBPα-mediated inhibition of this promoter, similar to observations made with DACH1, instead TCERG1 blocked the inhibition produced by C/EBPα. This, along with the finding that TCERG1 also abrogates the growth arrest activity of C/EBPα (a separate function from its transcription-related role) led to the labeling of TCERG1 as an inhibitor of C/EBPα rather than a co-repressor [Banman et al., 2010]. Analysis of mutants indicated that this inhibitory activity was located in the amino terminus of TCERG1 [Banman et al., 2010].

A potential mechanism for how TCERG1 inhibits C/EBPα was suggested by examination of the patterning of both proteins in the nucleus. C/EBPα localizes to pericentromeric regions, while TCERG1 partitions primarily to nuclear speckles. Interestingly, upon ectopic expression of C/EBPα, TCERG1 becomes relocalized to the pericentromeric regions [Banman et al., 2010; Moazed et al., 2011]. Moreover, analysis of several TCERG1 mutants showed a tight correlation between inhibitory activity toward C/EBPα and relocalization activity [Banman et al., 2010]. Most significantly in terms of the present paper, it is noteworthy that the C/EBPα inhibitory activity and the relocalization activity appear to both reside in the amino terminus of TCERG1.

In order to further our understanding of the mechanism whereby TCERG1 inhibits C/EBPα, we have pursued in the present study the identification of the motif(s) in TCERG1 that confers inhibition of C/EBPα and relocalization from nuclear speckles to pericentromeric regions. Our data suggest that the QA repeat domain is required for both of these activities.

MATERIALS AND METHODS

CELL LINES

COS7 and HEK293 cells were cultured in DMEM that was supplemented with 17% glucose and 10% fetal bovine serum.

PLASMIDS

The -68FX4-Luc reporter plasmid, pAdTrack-C/EBPα, and expression plasmids for T7-TCERG1 (originally referred to as pBOST7-CA150), mCherry-TCERG1, and EGFP-C1-C/EBPα, have all been previously described [Sune and Garcia-Blanco, 1999; McFie et al., 2006; Banman et al., 2010]. An expression plasmid for FLAG-C/EBPα was constructed which had the FLAG-epitope fused to the amino terminus of rat C/EBPα. Plasmids expressing T7-TCERG1 mutants with the QA repeat domain deleted or shortened (ΔQA, QA_{11}, and QA_{17}) were generated from pBOST7-TCERG1 by site-directed mutagenesis. Corresponding mCherry-TCERG1 mutants (ΔQA, QA_{10}, and QA_{20}) were generated from the pmCherry-TCERG1 parental plasmid. pmNeptune2-C1 and pmOrange2-C1 were obtained from Addgene and Clontech, respectively, and were used to generate expression plasmids for fluorescent protein fusions Neptune-WT-TCERG1and Orange-ΔQA-TCERG1. The plasmid expressing mCherry-FLAG-NLSX3-QA was generated starting with pmCherry-C1 and ligating to it in-frame coding regions for the FLAG epitope, three copies of the nuclear localization signal from the SV40 large T antigen, and the entire QA repeat domain.

QUANTIFICATION OF NUCLEAR SPECKLE QUANTITY AND SIZE

The transfection of COS7 cells, immunostaining, and laser-scanning confocal microscopy were performed according to procedures outlined in Moazed et al. [2011] except that the lipoD293 transfection reagent (FroggaBio) was used. Transfections were performed in 35 mm culture dishes using 0.45 μg of either mCherry TCERG1 ΔQA or mCherry TCERG1 WT and 0.55 μg of pTZ19R for a total of 1 μg of plasmid per well. The control group used 1 μg of pTZ19R. The primary antibody used for endogenous SC35 detection was MαSC35 (catalogue # ab11826, Abcam) diluted 1:1,000 in 3% BSA in PBS, and the secondary antibody was Alexa Fluor® 405 goat anti-mouse IgG (catalogue #A31553, Life Technologies) diluted 1:1000 in 3% BSA in PBS.

Imaging of cells was performed using a Zeiss LSM 700 confocal microscope under 63x magnification. Successfully transfected cells were confirmed by viewing the red fluorescence of mCherry, and only cells expressing mCherry were analyzed. Cell images were captured using a 2.5 μm Z-stack function with Zen Black software (Zeiss). Images were analyzed using ImageJ. The Z-stack was scanned for speckles (delineated by SC35 immunostaining) and the area of all speckles within 15 cells per treatment were measured using the elliptical function of ImageJ. The Z-stack was subsequently scanned to determine the number of speckles. Results were subjected to a standard two-tailed statistical test with a 95% confidence value.

GROWTH ARREST ASSAY

Growth arrest assays were performed in COS7 and HEK293 cells as previously described [McFie et al., 2006]. Cells were transfected on day 1, then 18–20 h later they were passaged to ~10–20% confluency via trypsinization and vigorous trituration to minimize cell clusters. Plates were observed on day 3 and assessed for numbers of green fluorescing cells presenting as clusters or single cells.

CO-IMMUNOPRECIPITATION AND WESTERN BLOTTING

HEK293 cells were transfected using 42 μg Polyethylenimine "MAX" MW 40,000 (Polysciences, Warrington, PA) and 5 μg of the T7-TCERG1 expression plasmid along with 2 μg of FLAG-N1-C/EBPα or pTZ19R in 100 mm culture dishes. The DNA: polyethylenimine mixture was incubated with 308 μL of 150 mM NaCl at RT for 20 min and then added to each plate containing 10 mL of complete media. Following incubation, the mixture was added to the cell culture well containing media and incubated at 37°C for 4–5 h. Subsequently, the media was replaced with fresh complete media and cells were incubated for 48 h. Cells were harvested at 4°C in binding buffer containing 25 mM HEPES pH 8.0, 0.5% Nonidet

P-40, 200 mM KCl, 1 mM EDTA, and 1X cOmplete® protease inhibitor cocktail (Roche). Crude lysate was subjected to fine needle aspiration and then incubated under gentle agitation for 1 h, followed by centrifugation at 10,000*g* for 30 min. Input samples were collected at this time. The supernatant was then pre-cleared using 50 μL Sepharose beads (Sigma-Aldrich) for 1 h under gentle agitation, then centrifuged for 20 min at 10,000*g*. The supernatant was incubated using 10 μL anti-FLAG magnetic beads (Sigma-Aldrich) for 2 h at 4°C. Flow-through samples were collected and the bead fractions were washed 5X with binding buffer. The beads were boiled in SDS–PAGE loading buffer for 5 min. Samples were analyzed by Western blot analysis using 1:10,000 rabbit anti-T7 HRP antibody (catalogue # A190-108P, Bethyl Laboratories) or 1:10,000 primary rabbit anti-C/EBPα (catalogue # sc-61, Santa Cruz) in conjunction with 1:10,000 secondary goat anti-rabbit HRP (catalogue # sc-2004, Santa Cruz) in 5% milk in PBST.

TRANSFECTION, IMMUNOSTAINING OF SC35, AND CONFOCAL MICROSCOPY

Transfection of COS7 cells grown on coverslips, fixation with paraformaldehyde, and permeabilization of cells with Triton X-100 was performed as described previously [Moazed et al., 2011]. SC35 was immunostained as described above. Coverslips were affixed to glass slides using Prolong® Diamond antifade reagent (Life Technologies).

Imaging was performed using a Leica SP5 confocal microscope at the Western College of Veterinary medicine, or a Zeiss LSM 700 confocal microscope provided by Dr. Deborah Anderson and Dr. Erique Lukong (College of Medicine, University of Saskatchewan). Images were obtained using Zen black edition (Zeiss) and further manipulated using FIJI image editing software (Fiji.sc).

RESULTS

Previous work from our laboratory indicated that the motif within TCERG1 that conferred inhibition of the growth arrest activity of C/EBPα resided in the amino terminus (amino acids 32-668, Fig. 1) [Banman et al., 2010]. This same fragment retained the ability to be translocated from nuclear speckles to pericentromeric DNA in response to ectopic C/EBPα expression. Interestingly, this region overlaps significantly with the original TCERG1 clone that we pulled-out from the yeast two-hybrid screen used to identify C/EBPα interactors, which coded for amino acids 89-480 (Fig. 1) [McFie et al., 2006].

The region extending from amino acids 32-668 contains a number of potential functional and/or structural motifs as shown in Figure 1. Initially, we focused on the WW domains, since they bind to proline-rich sequences, several of which are present in C/EBPα. Versions of TCERG1 with the WW1, WW2, or WW3 domains mutated [Goldstrohm et al., 2001] were tested for their ability to inhibit C/EBPα-mediated growth arrest of COS7 cells. All three WW mutants showed similar inhibitory activities compared to wild type TCERG1, as did a WW1,2 double mutant (data not shown). We subsequently turned our attention to another domain present in the original two-hybrid clone, the QA repeat domain.

Deletion of the QA domain (generating the ΔQA mutant) resulted in a significant loss of inhibitory activity toward the growth arrest activity of C/EBPα when assessed in COS7 cells. As shown in Figure 2A, expression of C/EBPα alone resulted in 90% of cells being in growth arrest (10% proliferating), which was almost completely reversed by co-expression of full-length TCERG1 (QA_{38}), consistent with our previous observations [McFie et al., 2006; Banman et al., 2010]. The ΔQA mutant, however, lost the ability to reverse C/EBPα-induced growth arrest. The loss of repressive activity was also observed in HEK293 cells (Fig. 2B). In order to assess whether all or only a fraction of the 38 QA repeats were necessary for inhibitory activity, we created two additional mutants possessing 17 and 11 repeats, respectively. As shown in Figure 2A and B, QA_{17} retained repressive activity similar to full-length TCERG1 while QA_{11} showed a loss of activity, similar to the ΔQA mutant.

We next examined the role of the QA domain on the interaction between C/EBPα and TCERG1. FLAG-C/EBPα was co-expressed in HEK293 cells with T7-tagged TCERG1 (and related mutants) and co-immunoprecipitated with anti-FLAG antibodies linked to agarose beads. The input lanes indicated that similar expression of the T7-taggged TCERG1 proteins and C/EBPα was obtained (Fig. 3). In the co-IP lanes, a significantly larger amount of TCERG1 WT and the QA_{17}

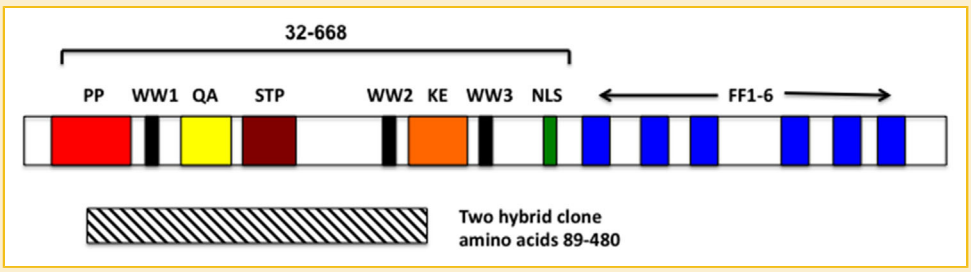

Fig. 1. Schematic of established and putative functional domains of TCERG1. The relative position of each domain is shown. Below the schematic is a diagonally hatched box showing the region of TCERG1 coded for the original clone pulled out of a two-hybrid screen using the transactivation domain of C/EBPα (amino acids 6–217) as the bait. The region of TCERG1 extending from amino acids 32–668 was that reported in Banman et al. [2010] to mediate both inhibition of C/EBPα–dependent growth arrest and relocalization of TCERG1. PP, polyproline region; QA, glutamine:alanine repeat region; STP, serine/threonine/proline rich region; KE, lysine/glutamate rich region; NLS, nuclear localization signal. The three WW and six FF domains are well-established protein interaction motifs.

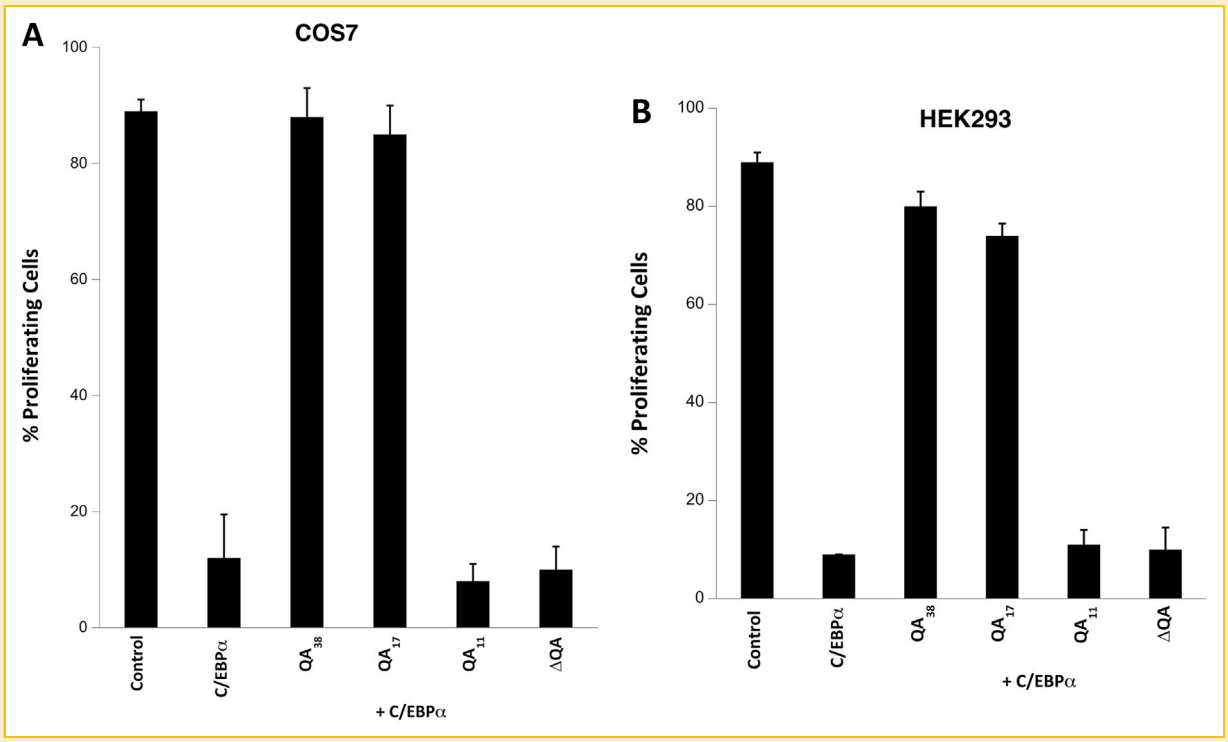

Fig. 2. Deletion or sufficient shortening of the QA repeat domain of TCERG1 abrogates its ability to inhibit C/EBPα-mediated growth arrest. Growth arrest assays were performed in either COS7 (panel A) or HEK293 (panel B) cells as described in the Materials and Methods Section. Cells transfected with empty pAdTrack and pBOST7 plasmids served as the control. C/EBPα was expressed from pAdTrack-C/EBPα which also expressed GFP. The four versions of TCERG1 that were co-expressed with C/EBPα had either the full complement of QA repeats (QA_{38}), 17 or 11 repeats (QA_{17} and QA_{11}), or a complete deletion of the QA domain (ΔQA). The data were expressed as the percent of green fluorescent cells present in clusters (i.e., proliferating), with the values representing the mean ± SE of three independent experiments.

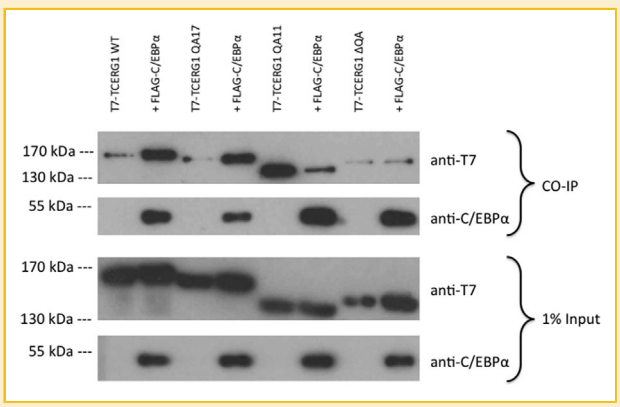

Fig. 3. Complex formation between C/EBPα and TCERG1 requires the QA repeat domain. HEK293 cells were transfected with plasmids expressing the indicated T7-tagged version of TCERG1 in the absence or presence of an expression plasmid for FLAG-C/EBPα. Co-immunoprecipitation was subsequently performed using anti-FLAG magnetic beads followed by Western blotting with either anti-T7 or anti-C/EBPα antibodies. The bottom two panels show relative expression levels of T7-tagged versions of TCERG1 and C/EBPα in the extracts used for co-immunoprecipitation.

mutant was present in the immunoprecipitate when FLAG-C/EBPα was co-expressed. However, when the QA domain was reduced to 11 repeats (QA_{11}) or deleted altogether (ΔQA), the amount of TCERG1 protein in the immunoprecipitate was not increased by FLAG-C/EBPα co-expression. It should be noted that the QA_{11} mutant exhibited a high background signal that was not increased when FLAG-C/EBPα was co-expressed (Fig. 3, Co-IP panel). Additionally, the ΔQA mutant consistently showed a higher apparent molecular weight compared to the theoretical expected size.

The ability of the QA repeat mutants to be relocalized from nuclear speckles to pericentromeric DNA by C/EBPα was explored. Initially, mCherry-tagged TCERG1 proteins were assessed for their localization to nuclear speckles in the absence of C/EBPα expression, by comparing with the localization of SC35, a well-described splicing factor that resides in nuclear speckles. As shown in Figure 4, the mCherry-TCERG1 WT and all three related mutants localized to nuclear speckles as evidenced by their co-localization with endogenous SC35 (Fig. 4, merged panel). Alterations in the nuclear speckle compartment were also consistently observed in the cells ectopically expressing TCERG1 or the ΔQA mutant (Table I). When either version of TCERG1 was over-expressed, an increase of about 30% in the number of speckles was observed relative to control cells transfected with empty vector. Moreover, relative to either control cells or when TCERG1 WT was ectopically expressed, the size of

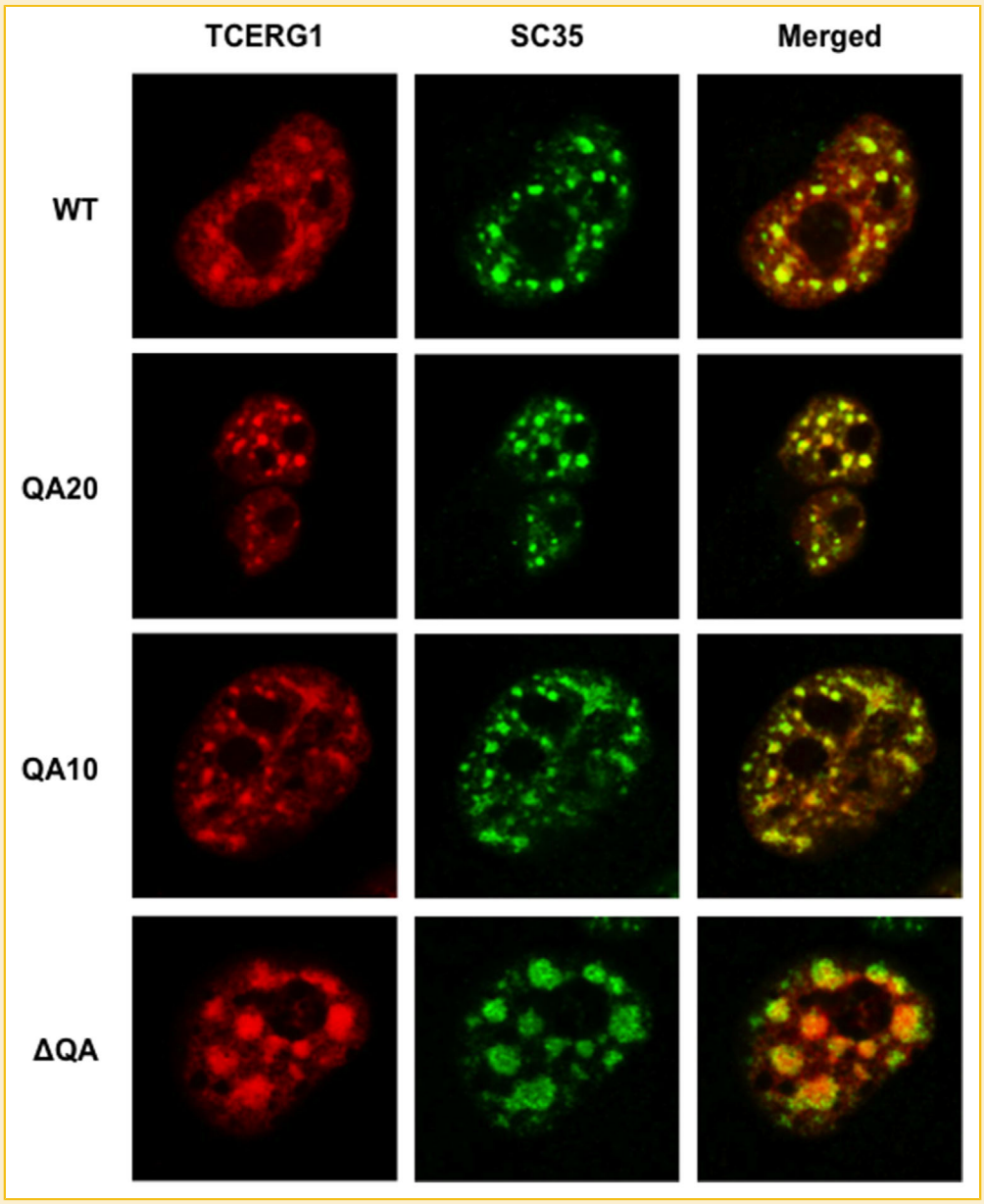

Fig. 4. Deletion of the QA domain does not affect the localization of TCERG1 to nuclear speckles. COS7 cells were transfected with an expression vector for the full-length or variant mCherry-TCERG1 fusion protein as indicated. Immunostaining of SC35 was performed prior to the mounting of coverslips on slides for confocal analysis. The merged panels were obtained by overlaying the TCERG1 and SC35 signals.

individual speckles was enlarged by 24% and 17%, respectively, when the ΔQA mutant was expressed (Table I). This was also occasionally observed with the mCherry-tagged QA_{10} mutant (see Fig. 5).

Consistent with previous findings [Banman et al., 2010], when mCherry-TCERG1 WT was co-expressed with EGFP-C/EBPα, the relocalization of TCERG1 from nuclear speckles to pericentromeric DNA where C/EBPα resides was observed (Fig. 5). This can be assessed not only from the merged panel of the mCherry and EGFP signals, but also from the merged panel of mCherry and SC35 which indicates that mCherry-TCERG1 was not co-localized with SC35 and thus no longer resident in nuclear speckles. Colocalization was also observed with the mCherry-tagged QA_{20} mutant. Conversely, the QA_{10} and ΔQA mutants of TCERG1 were not relocalized by co-expression of EGFP-C/EBPα (TCERG1-C/EBPα merged panel), but rather remained in the nuclear speckles as evidenced by the overlap seen in the mCherry and SC35 signals (mCherry and SC35 merged panel). Thus, the QA domain appears to be required for the ability of TCERG1 to be relocalized by C/EBPα.

We next addressed whether the QA domain is sufficient for relocalization. The QA domain was fused to mCherry to examine whether this fusion protein could be relocalized by C/EBPα. Since mCherry does not specifically partition to the nucleus [Banman

TABLE I. Ectopic Expression of a TCERG1 Mutant That Lacks the QA Repeat Leads to an Increase in the Size of Individual Nuclear Speckles

Experimental group	Nuclear speckle count	Nuclear speckle size (μM^2)
Control	27 ± 2	0.49 ± 0.03
TCERG1	35 ± 2^a	0.52 ± 0.03
ΔQA-TCERG1	35 ± 2^a	0.61 ± 0.04^a

COS7 cells were transfected with empty vector (control) or with an expression vector for either TCERG1 WT or TCERG1 ΔQA. After 48 h, cells were immunostained for SC35, and the average number and size and of individual nuclear speckles was determined as described in the Materials and Methods Section.

[a] $P \leq 0.05$ relative to control.

et al., 2010], three copies of a nuclear localization signal were fused to this chimeric protein. As seen in Figure 6 (top and bottom rows), the mCherry-QA fusion protein adopted irregular patterning in the nucleus and did not colocalize with SC35. Moreover, when co-expressed with EGFP-C/EBPα, no overlap was observed (bottom row). This suggests that the QA domain is unable to mediate co-localization with C/EBPα. The data also suggest that the QA domain is unable to act independently as a nuclear speckle localization signal.

Because the ΔQA mutant was unable to be relocalized from the nuclear speckles by C/EBPα, we examined whether this mutant could act in a dominant negative fashion to prevent full length TCERG1 from being relocalized. For this experiment, a far-red fluorescent protein, mNeptune2 was fused to TCERG1 WT while ΔQA TCERG1 was fused to mOrange2. Plasmids expressing these two fusion proteins were co-expressed along with EGFP-C/EBPα and visualized for their localization patterns by confocal microscopy. In Figure 7A, single channel images were obtained to visualize the localization patterns of the three fluorophores as well as immunostained SC35. In Figure 7B, various combinations of these single channel images were overlayed and are shown as merged images. The overlay of the WT and ΔQA TCERG1 images obtained when EGFP-C/EBPα was co-expressed showed a complete overlap in signal, suggesting that they co-localized. Overlay of the WT TCERG1 signal or the ΔQA TCERG1 signal with EGFP-C/EBPα indicate that both TCERG1 proteins localized to sites distinct from that of C/EBPα, indicating that the WT TCERG1 was not relocalized. Overlay of the WT and ΔQA TCERG1 signals with the SC35 signal indicated that the majority of both proteins remained localized to the nuclear speckles, further evidence that relocalization of TCERG1 WT did not occur. These data suggest that the ΔQA mutant can act in a dominant negative fashion to prevent relocalization of TCERG1 WT.

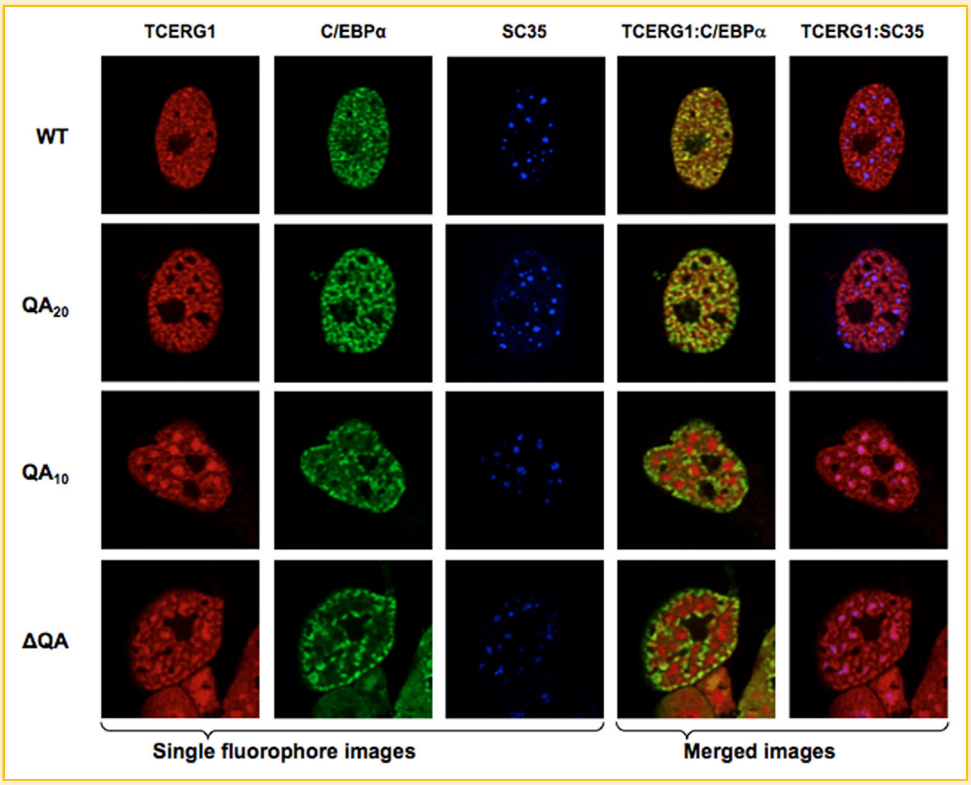

Fig. 5. Deletion of the QA domain abrogates the ability of TCERG1 to become relocalized to the pericentromeric regions by C/EBPα. COS7 cells were transfected with expression vectors for the mCherry-TCERG1 protein variant indicated on the left side of the figure, along with EGFP-C/EBPα. Immunostaining of SC35 was performed prior to the mounting of coverslips on slides for confocal analysis. The merged images in the last two columns were obtained by overlaying the indicated signals.

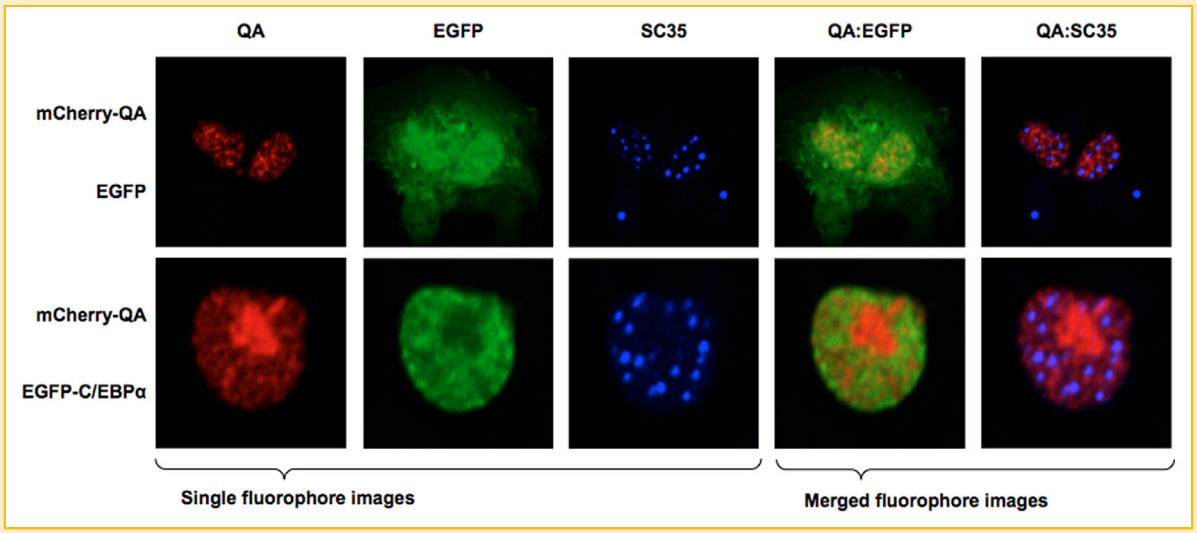

Fig. 6. The QA repeat domain is not able to confer relocalization ability when fused to a heterologous protein. COS7 cells were transfected with expression vectors for the mCherry-QA fusion protein along with either empty EGFP vector (top row) or the EGFP-C/EBPα vector (bottom row). Immunostaining of SC35 was performed prior to the mounting of coverslips on slides for confocal analysis. The merged images in the last two columns were obtained by overlaying the indicated signals.

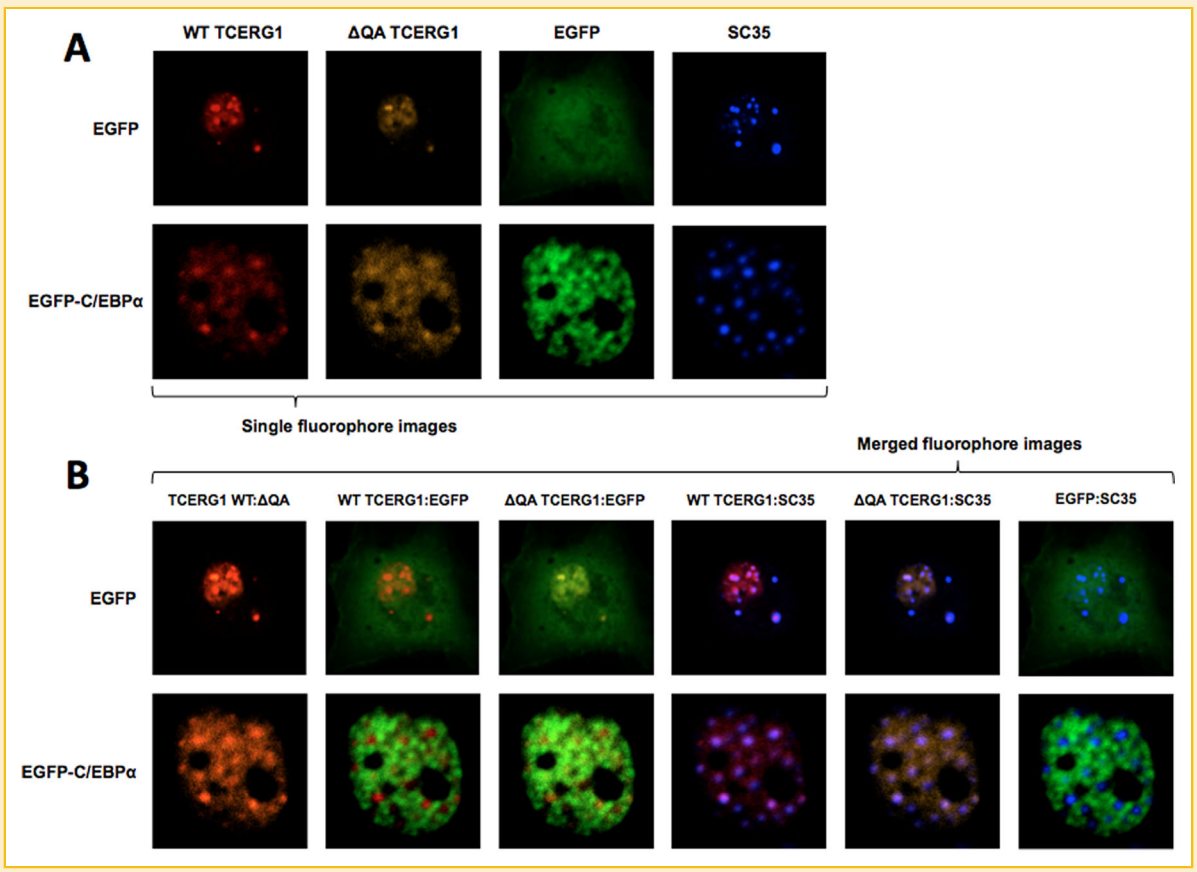

Fig. 7. The ΔQA mutant acts in a dominant negative fashion to block relocalization of full-length TCERG1. COS7 cells were transfected with expression vectors for the mNeptune-tagged WT TCERG1, mOrange-tagged ΔQA TCERG1, and EGFP-C/EBPα. Immunostaining of SC35 was performed prior to the mounting of coverslips on slides for confocal analysis. Individual fluorophore signals are shown in panel A, while panel B shows the various merged signals as indicated.

DISCUSSION

The overall goal of the present study was to identify the domain (s) in TCERG1 responsible for its inhibitory activity toward C/EBPα. Previous work from our laboratory demonstrated that TCERG1 inhibited not only the transcriptional activities of C/EBPα, but also its growth arrest activity [McFie et al., 2006; Banman et al., 2010]. We also showed that the ectopic expression of C/EBPα in cells led to the relocalization of TCERG1 from nuclear speckles to pericentromeric regions where C/EBPα is concentrated [Banman et al., 2010]. Mutants of TCERG1 that lacked the ability to be relocalized were also deficient in inhibitory activity toward C/EBPα [Banman et al., 2010]. The mutational analysis further suggested that residues 32-668, essentially the amino terminus half of the protein, contained the inhibitory domain as well as the relocalization domain. It was these observations that led us to explore this region further to localize the specific functional domains involved. The data presented in this paper suggest that the QA repeat domain is involved in both the interaction of TCERG1 with C/EBPα and in the inhibition of C/EBPα-mediated growth arrest.

The QA repeat domain in human TCERG1 consists of 35 QA repeats plus three QV repeats. Despite the unique nature of this motif, little is known or proposed about possible functions it might possess or participate in. In the one study that directly examined a role for this domain, Arango et al. [2006] showed that deletion of the QA domain resulted in the protein being retained in the cytosol, suggesting that the QA domain plays a role in nuclear localization. However, this appeared to be a striatal cell-specific effect, since they went on to show that in HEK293 cells, nuclear localization of the delta QA mutant still occurred which is consistent with the findings of Sanchez-Hernandez [Sanchez-Hernandez et al., 2012] and the present study. Holbert et al. [2001] identified TCERG1 as a potential interactor of the huntingtin protein in a yeast two-hybrid screen. While intriguing with respect to a possible link to Huntington's Disease, this study did not assign any specific biochemical function to the QA repeat motif.

The present study identifies an important role for the QA domain of TCERG1 in the repression of C/EBPα activity. That the QA domain participates in this repression is consistent with several observations made previously by our lab. The original TCERG1 clone pulled out in a yeast two-hybrid screen aimed at identifying interactors of C/EBPα, coded for amino acids 89-480 which encompasses the QA domain [McFie et al., 2006]. Furthermore, in a subsequent study we showed that the amino terminus half of TCERG1, coding for residues 32-668 which contains the QA motif, was sufficient for inhibition of the growth arrest activity of C/EBPα and was able to be relocalized in response to C/EBPα expression [Banman et al., 2010]. Our co-immunoprecipitation studies suggest a requirement for the QA domain in the interaction with C/EBPα, which may be sufficient for the relocalization of TCERG1 and inhibition of C/EBPα activity. This would be consistent with the observed loss of TCERG1's ability to interact with C/EBPα upon deletion of the QA domain, as well as the loss of relocalization ability and inhibitory activity.

While TCERG1 homologs are present in most eukaryotes including *C. elegans*, the QA repeat domain appears to be primarily a mammalian-specific feature [Bohne et al., 2000]. TCERG1 from nematodes, zebrafish, and fruit flies all display a single QA/AQ while chickens have two repeats. It is in mammals where significantly expanded QA repeats are observed, ranging from 20 to 41 in number depending on the species. It is interesting to note that the QA repeat expansion in mammals coincides closely with the appearance of the highly conserved mammalian C/EBPα, which differs significantly in both length and amino acid sequence from that in non-mammals such as chickens and zebrafish. This allows for the speculation that the coincident appearance of the expanded QA repeat in TCERG1 and of mammalian C/EBPα may have created a new regulatory circuit. Ongoing studies in the lab to identify the domain in C/EBPα that mediates the interaction with TCERG1 may inform this possibility.

While the present study identifies a role for the QA domain in the inhibitory activity of TCERG1 toward C/EBPα, the precise mechanism whereby TCERG1 represses C/EBPα activity remains elusive. The fact that deletion of the QA domain abrogates formation of TCERG1:C/EBPα complexes suggests that the effect of deleting the QA domain on the relocalization of TCERG1 and the ability of TCERG1 to inhibit C/EBPα may simply be a consequence of the loss of complex formation. In terms of how the interaction of TCERG1 with C/EBPα results in repression of both activities of C/EBPα, we previously proposed that TCERG1 may prevent the release of C/EBPα from pericentromeric DNA, thereby keeping it sequestered and thus functionally and physically unavailable for duty in the nucleus [Moazed et al., 2011]. However, we showed through the use of a C/EBPα mutant that demonstrates poor binding to pericentromeric DNA and thus is dispersed in the nucleus [Liu et al., 2007], that TCERG1 is still able to repress its activity and still co-localizes with the dispersed C/EBPα. An alternative possibility is that TCERG1 competes with a co-activator for binding to C/EBPα; while this might explain the ability of TCERG1 to inhibit the transactivation ability of C/EBPα, it would not appear to explain how this would lead to inhibition of the growth arrest activity of C/EBPα. It is possible that TCERG1 inhibits both activities of C/EBPα by two distinct mechanisms that share the need for relocalization of TCERG1. In support of this hypothesis, we have shown that the QA mutant of TCERG1 retains the ability to inhibit C/EBPα-mediated transactivation (unpublished data). This suggests that different domains are required for inhibition of growth arrest and transactivation activities of C/EBPα. Studies are underway to identify the domain(s) of TCERG1 that mediate the inhibition of the transactivation property of C/EBPα.

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