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ARTICLES

Cellular Transformation of NIH3T3 Fibroblasts by CIZ/NMP4 Fusions

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Abstract Molecular cloning of the translocations t(12;22)(p13;q12) and t(12;17)(p13;q11) in acute leukaemia showed that either *EWSR1* or its homologue *TAF15* are fused to the transcription factor *CIZ*. EWSR1 and TAF15 belong to the TET family (TLS/FUS, EWSR1 and TAF15) of proteins. TET fusions have been identified in both solid tumours and acute myeloid leukaemia. The novel 12p translocations directly implicated TET fusions in acute lymphoblastic leukaemia as well, and demonstrated the involvement of *CIZ* in haematopoietic malignancies. In addition, a new fusion *E2A-CIZ* was recently cloned as a result of a t(12;19)(p13;p13) in a patient with acute lymphoblastic leukaemia. NIH3T3 cells stably expressing TET-CIZ fusions display a transformed phenotype in a focus formation assay. We show here that E2A-CIZ also transforms 3T3 fibroblasts, suggesting that the addition of a transactivation domain to the CIZ protein is involved in this phenotype. An artificial *VP16-CIZ* construct reveals similar transforming properties, supporting this. We have then analysed the domains within TAF15-CIZ that are necessary for 3T3 fibroblast transformation. Deletion of the zinc fingers of CIZ resulted in loss of both DNA-binding and transforming properties of TAF15-CIZ, whereas deletion of the other functional domains of CIZ had no effect. Fusion of a transactivation domain to CIZ is suggestive for a transactivating function in transformation. Luciferase experiments indeed showed that E2A-CIZ as well as VP16-CIZ transactivates the *MMP7* promoter. Taken together, our results reported here suggest that transformation of 3T3 fibroblasts by CIZ fusions is dependent on DNA-binding and might involve transactivation of CIZ target genes. J. Cell. Biochem. 94: 1112–1125, 2005.

Key words: *CIZ* fusions; structure-function analysis; cellular transformation; transactivation; DNA-binding

The *TET-ETS* fusions are tumour-associated chromosomal rearrangements, in which the *EWSR1* (Ewing sarcoma1) gene or its homologues *TLS/FUS* (Translocated in LipoSarcomas) and *TAF15* (TATA box binding protein-associated factor 15) are fused to a member of the *ETS* (erythroblastosis virus-transforming sequence) family of transcription factors [Arvand and Denny, 2001]. *TET* fusions have been identified in both solid tumours and acute myeloid leukaemia. We recently cloned two chromosomal translocations t(12;17)(p13;q11) and t(12;22)(p13;q12) in acute lymphoid and myeloid leukaemia (ALL and AML), and showed that the rearrangements fuse the transcription factor *CIZ* (Cas-interacting zinc finger protein) to *TAF15* or *EWSR1*, respectively [Martini et al., 2002]. These recurrent translocations implicate *TET* fusions in lymphoid malignancies as well.

The TET proteins share an N-terminal transactivation domain and a unique C-terminal RNA-binding domain. It is assumed that TET proteins serve as a bridge between transcription and mRNA processing through their interaction with components of the transcription apparatus and splicing factors, respectively [Bertolotti et al., 1996, 1998; Yang et al., 1998; Meissner et al., 2003].

Rat *CIZ* was initially identified as a novel ligand for the $p130^{cas}$ docking protein, an important component of the integrin receptor signalling pathway [Nakamoto et al., 2000]. Feister and colleagues isolated the same

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protein as a nuclear matrix architectural transcription factor, and named it NMP4 (nuclear matrix protein isoform 4) [Feister et al., 2000]. An extensive range of *CIZ/NMP4* isoforms was characterised [Nakamoto et al., 2000; Thunyakitpisal et al., 2001]. They all derive from one single gene, and contain five to eight zinc fingers. Their multifunctional nature as well as nuclear and cellular partitioning can be regulated by alternative splicing [Bidwell et al., 2001]. Therefore, CIZ/NMP4 represents a novel family of nuclear matrix transcription factors that might transduce changes in cytoskeletal organisation into alterations in gene expression. CIZ upregulates the promoter activity of *MMP1*, *MMP3*, *MMP7* and *MMP13* (matrix metalloproteinases) [Nakamoto et al., 2000; Shah et al., 2004] as well as of the type-1 collagen (*COL1A1*) promoter [Alvarez et al., 1997; Furuya et al., 2000; Thunyakitpisal et al., 2001]. Furthermore, it is known to be implicated in bone metabolism, and acts as a novel type inhibitor of the bone morphogenetic protein/Smad signalling pathway [Shen et al., 2002]. In addition, recent studies of $CIZ^{-/-}$ mice demonstrate that CIZ might be involved in the progress and maintenance of spermatogenesis [Nakamoto et al., 2004]. Involvement of *CIZ* in acute leukaemia suggests that human CIZ might play a role during haematopoiesis as well.

The TET fusions are characterised by a common structure in which the C-terminal RNA-binding domain of the TET proteins is replaced with a transcription factor or its DNA-binding domain. At present, there is not a single mechanism that explains the full oncogenic potency of the TET fusions. They promote oncogenesis by acting as aberrant transcription factors. TET-ETS proteins predominantly localise to the nucleus in NIH3T3 cells overexpressing the fusions [Olsen and Hinrichs, 2001]. In addition, the N-terminal part of TET proteins encodes a more potent transactivation domain than the native one present in the transcription factors involved [Ohno et al., 1993; Bailly et al., 1994; Mao et al., 1994; Bertolotti et al., 1999]. Moreover, structure-function studies showed that other strong transactivation domains can functionally replace the N-terminal part of *TET* fusion genes [Lessnick et al., 1995]. Finally, representation difference analysis and microarray studies allowed the identification of a number of deregulated target genes [Braun et al., 1995; Lessnick et al., 2002]. Nevertheless, the full oncogenic potential of TET fusions appears to involve disruption of other cellular processes as well, such as mRNA splicing [Yang et al., 2000a,b]. Indeed, aberrant splicing products are frequently found in cancer cells, including those of Ewing's sarcoma [Hinohara et al., 1998; Barr et al., 1999]. In this regard, TET family members normally bind to RNA Pol II via their N-terminus and recruit various splicing factors through their C-terminal part. While the N-terminal domain is retained in TET fusions and still binds to RNA Pol II, the C-terminal domain fails to recruit splicing factors because of the replacement by the fusion partner. As a result, TET fusion proteins might block mRNA splicing, leading to degradation of the unprocessed pre-mRNA or generation of aberrant splicing products. For example, splicing of CD44, a cell-adhesion molecule whose abnormal splicing is associated with tumour cell growth and metastasis, was affected by TLS-ERG [Yang et al., 2000b]. Another DNA-independent mechanism, affecting human telomerase reverse transcriptase (hTERT) expression, has also been demonstrated [Takahashi et al., 2003; Fuchs et al., 2004], and could be involved in part of the oncogenic potency of TET fusions.

Hunger and colleagues recently identified a new fusion *E2A-CIZ* as a result of a cryptic t(12;19)(p13;p13) in ALL, in which the first transactivation domain of E2A is fused to the full-length CIZ [Hunger and Zhong, 2002]. This suggests that aberrant transactivation is indeed involved in transformation by CIZ fusion proteins. Here, we performed a structure-function analysis to define the domains within TAF15-CIZ that are essential for transcriptional regulation and transformation.

MATERIALS AND METHODS

Plasmid Construction

For the construction of the *TAF15-CIZ* deletion mutants and the novel *CIZ* fusions, we used as template DNA *TAF15-CIZ* or wild-type *CIZ*, both containing all eight zinc fingers present in CIZ [Martini et al., 2002]. The *TAF15-CIZ* deletion mutants were created by a two-step PCR (primers: overview Table I), using the Pfu polymerase (Stratagene). In brief, the respective N-terminal parts were amplified with the common 5′ primer TAF15-CIZ-F, containing a *BglII* site, and a reverse primer with a unique

TABLE I. PCR Primers Used for Plasmid Construction

Primer	Sequence[a]	RE
VP16-CIZ-F	5′-CG*AGATCT*GCCACCATGGCCCCCCCGACCGATGTC	*BglII*
VP16-CIZ-R	5′-CATTCT*GCCGGC*CCCACCGTACTCGTCAAT	*NaeI*
E2A-CIZ-F	5′-G*AGATCT*GCCACCATGAACCAGCCGCAGAGG	*BglII*
E2A-CIZ-R	5′-CATC*CGGCCG*AGGAGGCTGTCGGCCCCGCT	*EagI*
CIZ-EagI-F	5′-CTCCT*CGGCCG*GATGGAAGAATCTCACTTC	*EagI*
CIZ-F	5′-G*AGATCT*AATGCCGGCAGAATGGAAGAATCTCACTTC	*BglII*
TAF15-CIZ-F	5′-G*AGATCT*GCCACCATGTCGGATTCTGGAAGT	*BglII*
TAF15-CIZ del LZ-F	5′-TGGCC*GTCGAC*AGTGCCTGCCTCAGTGTCC	*SalI*
TAF15-CIZ del LZ-R	5′-CACT*GTCGAC*GGCCAGAAGTACGGGTTAGA	*SalI*
TAF15-CIZ del SR-F	5′-GTGC*CGGCCG*AGGAAGGAGGCGGAGGTGGT	*EagI*
TAF15-CIZ del SR-R	5′-TTCCT*CGGCCG*GCACTGTCAGCAAGGTGGG	*EagI*
TAF15-CIZ del PR-F	5′-AGTGT*CGGCCG*GAAGAAGAAGCGGATGCTG	*EagI*
TAF15-CIZ del PR-R	5′-CTTC*CGGCCG*ACACTGCCACCTCCACCACCA	*EagI*
TAF15-CIZ del NLS-F	5′-CCAC*CTCGAG*GCATGCTGGAATCAGGGCTGCC	*XhoI*
TAF15-CIZ del NLS-R	5′-CATGC*CTCGAG*GTGGCTTAGGAGCCACACT	*XhoI*
TAF15-CIZ del ZF-F	5′-ACCTAC*CGGCCG*CCTGATCTTCAGCAACAG	*EagI*
TAF15-CIZ del ZF-R	5′-AGG*CGGCCG*GTAGGTCTTGCCGTCTTTCTG	*EagI*
TAF15-CIZ del QA-F	5′-GCAGT*GGCGCC*ACCACCACACTTCCAGTCT	*NarI*
TAF15-CIZ del QA-R	5′-TGGT*GGCGCC*ACTGCTGCCGCTGCTGCTGC	*NarI*
CIZ-R	5′-A*GGGCCC*AGAGCTGGCCAGGTGCTCCA	*ApaI*

[a]Unique restriction enzymes (RE) introduced in the respective primers are in italic in the sequences and indicated at the right.

restriction site. For the C-terminal parts, the corresponding forward primers were used in combination with the common 3′ primer CIZ-R, containing an *ApaI* site. All PCR products were cloned in pGEM-Teasy (Promega), and sequenced. The *TAF15-CIZ* deletion mutants were then generated by a three-way ligation into pcDNA3.1-6HisMyc (Invitrogen), cut with *EcoRI*/*ApaI*. Finally, the pcDNA3.1 *TAF15-CIZ* deletion constructs were digested with *BglII*/*PmeI*, and transferred into the retroviral expression vector pMSCV-puro (Clontech), cut with *BglII*/*HpaI*. An in-frame 6HisMyc-tag was present at the 3′-end of all expression constructs.

The pMSCV *TAF15-CIZ* deletion constructs were then digested with *NaeI*/*EcoRI*, and the insert fragments were subcloned into pMSCV-puro, cut with *HpaI*/*EcoRI*, to create the corresponding *CIZ* deletion mutants. The *TAF15-CIZ* without the serine-rich region contains an internal *NaeI*, and therefore *CIZ del SR* was amplified by PCR from the *TAF15-CIZ del SR* with the primers CIZ-F and CIZ-R, incorporating a *BglII* site at the 5′- and an *ApaI* site at the 3′-end, respectively. This PCR product was cloned in pGEM-Teasy (Promega). The *BglII*/*ApaI* fragment is then transferred into the vector fragment pMSCV *TAF15-CIZ*, digested with *BglII*/*ApaI*, containing the 6HisMyc-tag for detection.

For the *VP16-CIZ* fusion, a cDNA fragment containing the VP16 transactivation domain (amino acids 413–498) was amplified by PCR from the SNATCHII vector [Alen et al., 1999], with the primers VP16-CIZ-F and VP16-CIZ-R, introducing a *BglII* site and the Kozak consensus sequence at the 5′-end, and a *NaeI* site at the 3′-end. The amplification product was cloned in pGEM-Teasy (Promega). *VP16 EcoRI*/*NaeI* and *CIZ NaeI*/*ApaI* were subcloned in pcDNA3.1-6HisMyc *EcoRI*/*ApaI*. For *E2A-CIZ*, the *E2A* portion (amino acids 1–319) was amplified by PCR from the Image clone 12327-M11 with the primers E2A-CIZ-F and E2A-CIZ-R, incorporating a *BglII* site at the 5′- and an *EagI* site at the 3′-end, respectively. PCR was performed with the primers CIZ-*EagI*-F and an internal primer to create an *EagI* site instead of a *NaeI* site at the 5′-end of *CIZ*. The *EcoRI*/*EagI* fragment of *E2A* is then fused to the *EagI*/*ApaI* fragment of *CIZ*, and subcloned in pcDNA3.1-6HisMyc *EcoRI*/*ApaI*.

Finally, both fusion constructs (*VP16-CIZ* and *E2A-CIZ*) were subcloned into pMSCV-puro as described for the *TAF15-CIZ* deletion constructs, retaining the 6HisMyc-tag.

Cell Culture, Virus Production and Transduction

HEK293T and NIH3T3 cell lines were cultured in DMEM-F12 medium (Life Technologies), supplemented with 10% fetal calf serum (Hyclone). Retroviral stocks were prepared by co-transfection of HEK293T cells with the respective pMSCV constructs and an ecotropic packaging plasmid (pIK6.1MCV), using Fugene-6 (Roche). Viral stocks (10^5–10^7 infec-

tious particles/ml) were collected 48 h post-transfection and used to transduce NIH3T3 fibroblasts in the presence of polybrene (8 μg/ml) (Sigma). After 24 h, selection with puromycin (2 μg/ml) was started. Experiments were set up within 2 weeks after transduction, and were performed at least in duplicate using independent transductions. For the focus formation assay, NIH3T3 cells stably expressing the different constructs were seeded at a density of 10^6 cells per 9 cm culture dish and cultured for 3 weeks. Foci appeared 10–14 days after transduction. Macroscopic pictures were obtained after staining with 1% crystal violet in phosphate-buffered saline (PBS). In addition, cultures of cells overexpressing one of the constructs were trypsinised at confluency after 3 weeks of culture and counted using a Coulter Multisizer.

Immunofluorescence

For subcellular localisation of the proteins, 2×10^4 transduced NIH3T3 cells per well were plated in an 8-chamber slide. Alternatively, 10^4 HEK293T cells per well were transfected with Fugene-6 (Roche). Cells were grown to 60–70% confluency, and then fixed with 4% paraformaldehyde for 20 min followed by two washes in PBS. Subsequently, cells were permeabilised for 10 min with 0.1% Triton-X 100 (Sigma) in PBS. After blocking for 30 min with PBS-1% BSA-10% goat serum (gs), antibodies, diluted in PBS–BSA-gs, were applied for 1 h at room temperature. The antibodies used are: anti-c-myc monoclonal antibody (clone 9E10) and goat anti-mouse Alexa fluor 488 (Molecular probes). All wash steps were performed with PBS–BSA. After post-fixation, slides were mounted with PBS-glycerol (Citifluor). Images were captured either by a digital imaging microscopy using a cooled charge-coupled device camera (Photometrics) with the Smart Capture software (Vysis) or by the laser scanning confocal imaging system MRC1024 (Bio-Rad) attached to an inverted microscope Diaphot 300 (Nikon) with the LaserSharp software (Bio-Rad).

Western Blot Analysis

Cells were lysed in 20 mM Tris pH 7.5, 200 mM NaCl, 1% NP40 (v/v), containing protease inhibitors (Complete, Roche). Cell lysate proteins (30–50 μg) were separated on 10% SDS polyacrylamide gels (Bio-Rad), transferred to Hybond-P membranes (Amersham Pharmacia) and incubated with the antibodies, using standard techniques. The anti-c-myc mouse monoclonal antibody (9E10) was kindly provided by J. Creemers (Department of Human Genetics, Leuven, Belgium). Peroxidase-conjugated sheep anti-mouse (Amersham Pharmacia) was used as secondary antibody.

Luciferase Reporter Assay

Reporter assays were performed in HEK293T cells, seeded in a 24-well plate and grown at 35% confluency prior to transfection. After 24 h, cells were co-transfected with a luciferase reporter construct containing the human matrilysin (*MMP7*) promoter (150 ng) [Crawford et al., 2001], together with one of the expression vectors and 15 ng of a β-galactosidase expression plasmid as internal control, using Fugene-6 (Roche). The total amount of transfected DNA was kept constant at 200 ng per well with empty vector DNA. After 36 h, cells were washed with PBS, lysed in 30 μl passive lysis buffer (Promega), and luciferase and β-galactosidase activities were measured with the FLUOstar Galaxy luminometer (BMG, Labtechnologies), using the assays from Promega and Tropix (Westburg), respectively. All luciferase values were normalised for β-galactosidase activity, and represent the average of at least three independent experiments; the error bars show S.E. values. To evaluate protein expression levels, equal volumes of total cell lysate were subjected to Western blot analysis.

Electrophoretic Mobility Shift Assay (EMSA)

HEK293T cells were seeded at 1.5×10^6 cells in a 9 cm culture dish, and transfected with the respective pMSCV plasmids, using Fugene-6 (Roche). After 24 h, nuclear extracts were prepared with the Nuclear Extract kit (Active Motif), according to the manufacturer's instructions. Approximately 20 μg of nuclear proteins was incubated with 50 ng poly(dI-dC) (Pharmacia) and constant amounts (~20,000 dpm) of labelled oligonucleotides in EMSA binding buffer (10 mM HEPES, 2.5 mM $MgCl_2$, 0.05 mM EDTA, 8.5% glycerol, 1 mM DTT, 0.05% Triton-X-100 and pH 7) on ice for 20 min, and subsequently subjected to non-denaturing polyacrylamide gel electrophoresis on 5% gels at 120 V for 90 min. The DNA probe was prepared by annealing the oligonucleotides 5′-CGC-GTCAACCTTTTTCAAAAAGACCAG and its complement, encoding the −320 to −305 region

of the human *MMP1* promoter, and labelled with [α-^{32}P]-dCTP by filling in 5′-overhangs with Klenow polymerase. The mutant probe was prepared by annealing oligonucleotides 5′-CCTGTGTCAGAGAGA and its complement. For competition and supershift experiments, unlabelled probe and anti-c-myc antibody, respectively, were added to the reaction mixture 5 min prior to the labelled probe.

RESULTS

CIZ Fusions Containing Heterologous Transactivation Domains Have Transforming and Transactivating Properties

The N-terminal part of TAF15 and EWSR1, present in the TET-CIZ fusions, functions as a transactivation domain [May et al., 1993; Lessnick et al., 1995]. Interestingly, a different translocation fusing the transactivation domain of E2A to CIZ was recently shown to be involved in ALL [Hunger and Zhong, 2002]. Since the TET-CIZ fusions showed transforming properties in 3T3 fibroblasts, we thus investigated whether the E2A-CIZ fusion is also able to transform these cells. NIH3T3 cells stably expressing E2A-CIZ clearly displayed a transformed phenotype, compared with cells overexpressing CIZ. The foci induced by E2A-CIZ mainly contained spindle-like cells (Fig. 2A). A similar transformed phenotype has been observed in 3T3 fibroblasts overexpressing E2A-HLF or E2A-PBX1 [Yoshihara et al., 1995; Kamps et al., 1996]. Subsequently, we investigated if the fusion of an unrelated strong transactivation domain to CIZ was sufficient for acquiring transforming properties in fibroblasts. Therefore, we fused the entire activation domain of VP16 (amino acids 413–498) to CIZ (Fig. 1C), since it was shown that the core VP16 (amino acids 416–454) stimulated transcription less efficiently [Triezenberg et al., 1988]. VP16-CIZ induced foci of small round cells, resulting in a different phenotype in comparison with E2A-CIZ (Fig. 2A). In addition, VP16-CIZ was more potent in fibroblast transformation than E2A-CIZ: the foci appeared earlier and became larger, resulting in a higher cell density after 3 weeks of culture (Fig. 2B). Western blot analysis confirmed expression of the recombinant proteins (Fig. 2C). The TET N-terminal domain appears to be functionally interchangeable between the different TET fusion proteins, whereas the DNA-binding domain, acquired by the fusions, defines the specific tumour phenotype [Thompson et al., 1999]. However, our results showed that the morphology of the foci was affected as a result of overexpressing either VP16-CIZ or E2A-CIZ, indicating that domains other than the transcription factor component can interfere, probably through interaction with cell-type-specific co-factors.

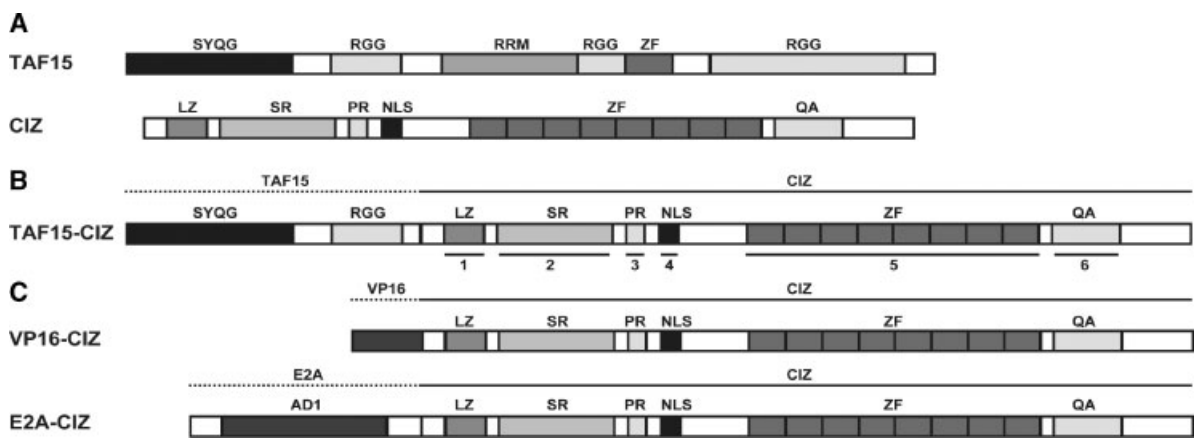

Fig. 1. Structure of the different CIZ and TAF15-CIZ fusions. **A**: Structure of the TAF15 and CIZ proteins. TAF15 contains a transactivation domain at the N-terminus, consisting of a series of degenerate repeats (SYQG), and an RNA-binding domain composed of three arginine-glycine-glycine rich (RGG) regions and an RNA recognition motif (RRM) at the C-terminus. A zinc finger (ZF) is flanked by two RGG boxes. CIZ contains leucine-rich (LZ), serine-rich (SR) and proline-rich (PR) domains at the N-terminus, followed by a putative nuclear localisation signal (NLS), eight Krüppel-type C_2H_2 zinc fingers (ZF) and a glutamine-alanine repeat (QA) at the C-terminus. **B**: Structure of TAF15-CIZ and the TAF15-CIZ deletion mutants. The TAF15-CIZ deletion mutants each lack one specific region of CIZ, indicated by the black bars: TAF15-CIZ del LZ (1), TAF15-CIZ del SR (2), TAF15-CIZ del PR (3), TAF15-CIZ del NLS (4), TAF15-CIZ del ZF (5), TAF15-CIZ del QA (6). **C**: Structure of the CIZ fusions containing heterologous transactivation domains. The transactivation domain of TAF15 is replaced with that of VP16 or E2A, fused to the full length CIZ.

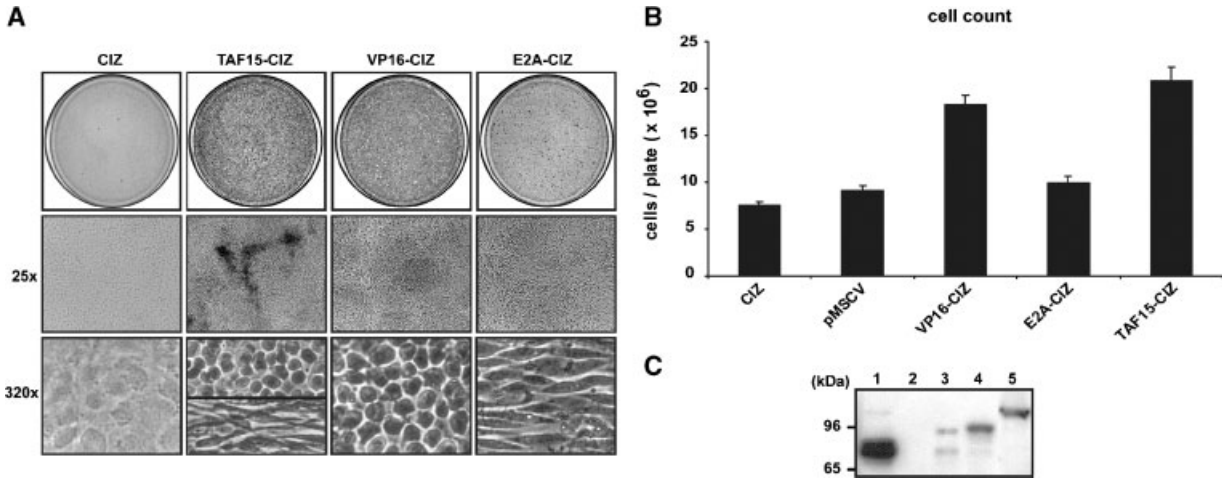

Fig. 2. Transforming properties of the CIZ fusions containing heterologous transactivation domains. **A**: Focus formation assay. NIH3T3 cells stably expressing CIZ, TAF15-CIZ, VP16-CIZ or E2A-CIZ were seeded at a density of 10^6 cells per 9 cm culture dish and cultured for 3 weeks. Foci appeared 10–14 days after transduction. Macroscopic pictures were obtained after staining with 1% crystal violet in PBS. Microscopic pictures were taken at two different magnifications, indicated at the left. For TAF15-CIZ, two distinct regions of the same dish are presented at highest magnification to illustrate both the spindle-like shape (lower) and the small round cell (upper) morphology. VP16-CIZ generated uniquely islands of round cells, whereas E2A-CIZ generated spindle-like cells. **B**: Cell density. Cultures of cells overexpressing CIZ, TAF15-CIZ, VP16-CIZ or E2A-CIZ were trypsinised at confluency after 3 weeks of culture and counted using a Coulter Multisizer. Both experiments were performed in duplicate, using independent transductions. **C**: Western blot analysis. Equal volumes of total cell lysate were separated on 10% SDS–PAGE and proteins were detected with anti-c-myc antibody. **Lane 1**, CIZ; **Lane 2**, pMSCV-puro; **Lane 3**, VP16-CIZ; **Lane 4**, E2A-CIZ; **Lane 5**, TAF15-CIZ.

Thus, when CIZ is fused to different heterologous transactivation domains, it acquires transforming properties in 3T3 fibroblasts. To further investigate if the fusions affect the transactivating properties of CIZ, we determined the transactivating activity of VP16-CIZ and E2A-CIZ fusions. Luciferase reporter assays were performed in HEK293T cells, transfected with a reporter construct containing the −301/+35 region of the human *MMP7* promoter, together with an internal control and the respective fusion constructs. VP16-CIZ significantly activated the *MMP7* promoter (Fig. 3A), inducing a five-fold increase of luciferase activity in comparison with empty vector. No strong transcriptional activity was measured for E2A-CIZ at similar expression levels, although transactivation was increased when higher amounts of *E2A-CIZ* DNA were used for transfection (Fig. 3A,B). These findings suggest that transcriptional activation might be one mechanism for cellular transformation of 3T3 fibroblasts mediated by CIZ fusions.

DNA-Binding and Transactivating Properties of TAF15-CIZ and CIZ Deletion Mutants

We and others demonstrated specific binding of CIZ to the human *MMP1* promoter [Nakamoto et al., 2000; Martini et al., 2002]. In addition, Torrungruang and co-workers showed that deletion of the zinc fingers abrogated specific DNA-binding [Torrungruang et al., 2002a]. Furthermore, EMSA studies indicated that only three specific zinc fingers of CIZ (ZFs 2, 3 and 6) are required for binding to the DNA consensus sequence. This is consistent with observations for other zinc finger proteins in which only two to four zinc fingers are necessary for DNA-binding, whereas additional zinc fingers define the affinity and specificity of binding or mediate protein–protein interactions [Mackay and Crossley, 1998; Wolfe et al., 2000]. To determine whether the zinc fingers contain DNA-binding properties in the CIZ fusions as well and to investigate the role of the other functional domains of CIZ, we created 6 myc-tagged *TAF15-CIZ* deletion mutants, each lacking one specific domain of CIZ (Fig. 1B). Nuclear extracts were prepared from HEK293T cells, transfected with the respective *TAF15-CIZ* deletion mutants. All TAF15-CIZ fusions were efficiently translated at approximately equal levels as revealed by Western blot analysis (Fig. 4B). Note that all TAF15-CIZ fusions migrate slower than predicted from their molecular weight. This may be due to the

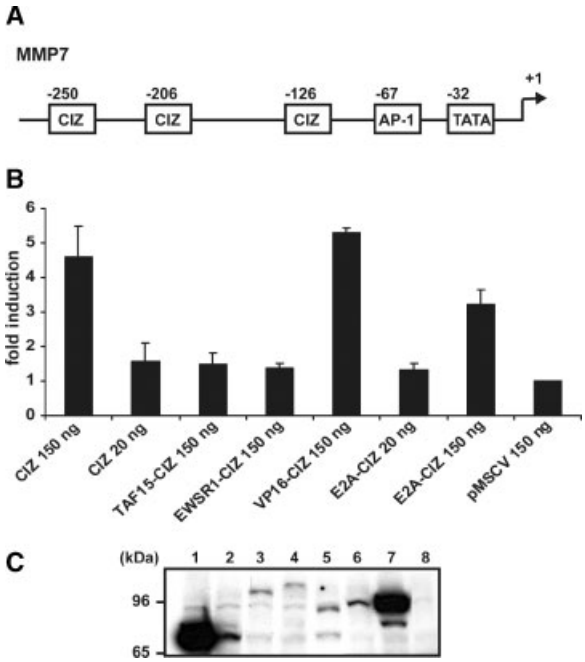

Fig. 3. Transactivating properties of the CIZ fusions containing heterologous transactivation domains. **A**: Structure of the human *MMP7* (matrilysin) promoter from −301/+35. Indicated are the positions relative to the transcriptional start site of three consensus CIZ binding sites as well as the positions of the *AP-1* site and the TATA box. **B**: Luciferase reporter assay. HEK293T cells were co-transfected with a human *MMP7* promoter (-296HMAT-Luc) reporter construct together with one of the expression constructs (DNA amount indicated) and a β-galactosidase expression plasmid. After 36 h, β-galactosidase and luciferase activities were measured. Luciferase values, normalised for β-galactosidase, are the means of three independent experiments with error bars representing the standard deviations. Luciferase activity in cells co-transfected with empty vector was set at 1. **C**: Western blot analysis. Equal volumes of total cell lysate were separated on 10% SDS–PAGE and proteins were detected with anti-c-myc antibody. **Lane 1**, CIZ (150 ng); **Lane 2**, CIZ (20 ng); **Lane 3**, TAF15-CIZ (150 ng); **Lane 4**, EWSR1-CIZ (150 ng); **Lane 5**, VP16-CIZ (150 ng); **Lane 6**, E2A-CIZ (20 ng); **Lane 7**, E2A-CIZ (150 ng); **Lane 8**, pMSCV-puro (150 ng).

high glycine, serine and glutamine content of the TAF15 portion present in the fusions. The expression level of CIZ as well as E2A-CIZ was higher than that of the TAF15-CIZ fusions, whereas VP16-CIZ expression was comparable. EMSAs were then performed with a ^{32}P-labelled double stranded oligonucleotide, containing the −320/−305 region of the human *MMP1* promoter, as probe. The TAF15-CIZ mutant lacking the zinc fingers completely lost affinity for the probe, confirming that the zinc fingers constitute a DNA-binding domain in the TAF15-CIZ fusion. All other TAF15-CIZ deletion mutants showed specific binding to *MMP1* (Fig. 4A). The nuclear extracts of TAF15-CIZ

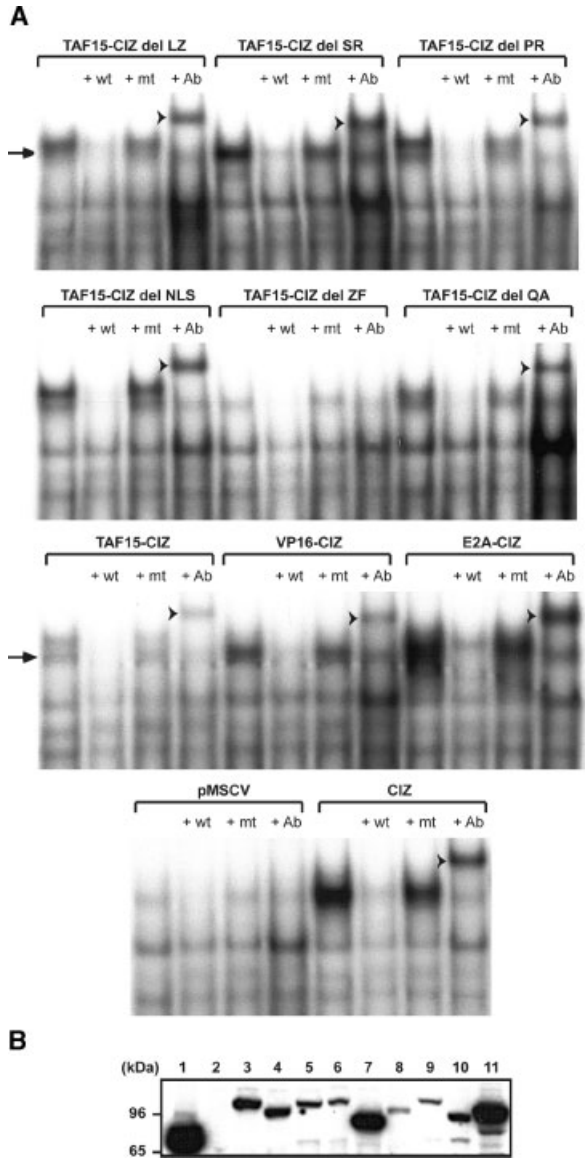

Fig. 4. DNA-binding properties of the TAF15-CIZ deletion mutants and CIZ fusions. **A**: EMSA. Nuclear proteins of HEK293T cells transfected with *CIZ, CIZ* fusions or *TAF15-CIZ* deletion mutants were capable of binding to *MMP1*, except for TAF15-CIZ del ZF. The binding was specific since the complex supershifted with anti-c-myc antibody (ab) and disappeared upon addition of 1,000-fold excess of cold wild-type probe (wt), but not when the same amount of cold mutant probe (mt) was used. Arrowheads, shifted complexes. Arrows, endogenous CIZ. **B**: Western blot analysis. Equal amounts of nuclear extract (∼40 μg of protein) were separated on 7.5% SDS–PAGE and protein expression was examined by Western blot analysis with anti-c-myc antibody. **Lane 1**, CIZ; **Lane 2**, pMSCV-puro; **Lane 3**, TAF15-CIZ del LZ; **Lane 4**, TAF15-CIZ del SR; **Lane 5**, TAF15-CIZ del PR; **Lane 6**, TAF15-CIZ del NLS; **Lane 7**, TAF15-CIZ del ZF; **Lane 8**, TAF15-CIZ del QA; **Lane 9**, TAF15-CIZ; **Lane 10**, VP16-CIZ; **Lane 11**, E2A-CIZ.

lacking the putative nuclear localisation signal also retained specific DNA-binding. This suggests that other sequences in CIZ must be responsible for nuclear targeting. VP16-CIZ as well as E2A-CIZ formed a specific gel shift complex, confirming the results from the reporter assays.

As discussed, it is not possible to measure significant transactivation of *MMP1* by the TAF15-CIZ fusion, due to low expression levels [Martini et al., 2002]. Since this experimental limitation does not apply to CIZ, we tested the transactivating properties of the corresponding CIZ deletion mutants. Luciferase reporter assays were performed in HEK293T cells. Expression of all CIZ mutants was confirmed by Western blot analysis (Fig. 5B). The two bands observed in CIZ may be due to internal initiation of translation, since the putative leucine zipper contains two alternative start codons and CIZ without the leucine zipper showed only one single band, whereas the other deletion mutants displayed a comparable pattern as wild-type CIZ. We observed a similar transactivation potential for all but CIZ without the zinc fingers (Fig. 5A). Deletion of the zinc fingers resulted in complete loss of transactivation. CIZ lacking the leucine zipper or the glutamine stretch were less active than wild-type CIZ, probably as a result of their lower expression. Alternatively, the glutamine stretch present in CIZ contributes to transactivation in the context of the native protein [Torrungruang et al., 2002a], and deletion of this domain might therefore reduce transactivating capacity of the CIZ protein. Our data show that the zinc finger domain is required for DNA-binding and transactivation.

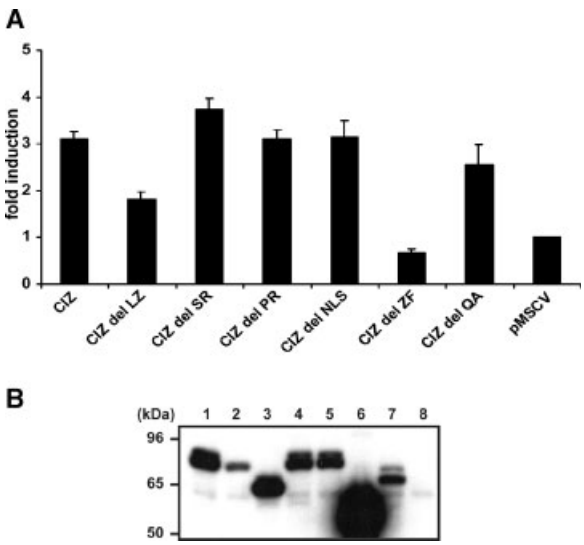

Fig. 5. Transactivating properties of the CIZ deletion mutants. **A**: Luciferase reporter assay. HEK293T cells were co-transfected with a human *MMP7* promoter (-296HMAT-Luc) reporter construct together with one of the expression constructs (150 ng) and a β-galactosidase expression plasmid. After 36 h, β-galactosidase and luciferase activities were measured. Luciferase values, normalised for β-galactosidase, are the means of two independent experiments with error bars representing the standard deviations. Luciferase activity in cells co-transfected with empty vector was set at 1. **B**: Western blot analysis. Equal volumes of total cell lysate were separated on 10% SDS–PAGE and proteins were detected with anti-c-myc antibody. **Lane 1**, CIZ; **Lane 2**, CIZ del LZ; **Lane 3**, CIZ del SR; **Lane 4**, CIZ del PR; **Lane 5**, CIZ del NLS; **Lane 6**, CIZ del ZF; **Lane 7**, CIZ del QA; **Lane 8**, pMSCV-puro.

Subcellular Localisation of the CIZ and TAF15-CIZ Deletion Mutants

Several studies illustrate that CIZ is mainly present in the nucleus [Feister et al., 2000; Nakamoto et al., 2000; Torrungruang et al., 2002b]. The *CIZ/NMP4* gene, however, encodes numerous isoforms [Nakamoto et al., 2000; Thunyakitpisal et al., 2001], which display distinct subcellular localisations. In addition to its nuclear localisation, CIZ/NMP4 isoforms are also present in the mitochondria, within or proximal to the Golgi apparatus, and faintly throughout the cytoplasm, observed in a variety of cell types [Torrungruang et al., 2002b]. Nevertheless, there is some controversy concerning whether these proteins are localised at focal adhesions [Furuya et al., 2000; Nakamoto et al., 2000; Torrungruang et al., 2002b]. It appears that the polyclonal antibodies used by the different groups likely recognise distinct isoforms. We studied the cellular distribution of CIZ, TAF15-CIZ and their corresponding deletion mutants in NIH3T3 as well as in HEK293T cells. Our results showed that subcellular localisation of CIZ was restricted to the nuclear compartments of the cell (Fig. 6A), confirming previous data from Feister and co-workers, who showed that all green fluorescent protein (GFP)-NMP4 fusion proteins localised to the nucleus, but accumulated in distinct nuclear subdomains [Feister et al., 2000]. Additionally, they demonstrated that a minimum of five zinc fingers (ZFs 1–5 or ZFs 4–8) was necessary and sufficient for exclusive nuclear localisation and association with the nuclear matrix. We further observed that the TAF15-CIZ fusion remained in the nucleus, as described for other TET fusion proteins [Olsen and Hinrichs, 2001]. By confocal

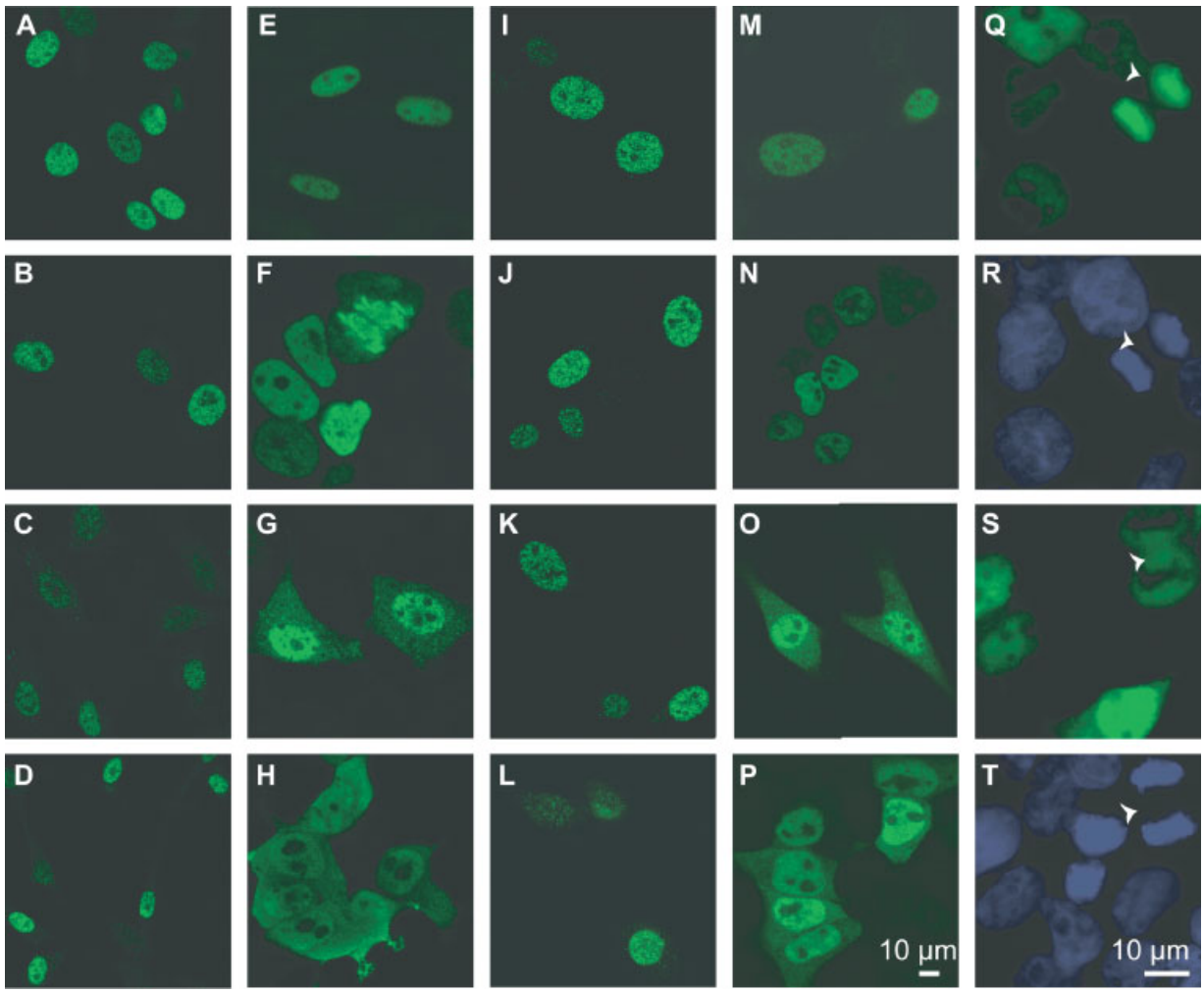

Fig. 6. Subcellular localisation of the CIZ and TAF15-CIZ fusions. NIH3T3 cells (or HEK293T cells when indicated) expressing CIZ, TAF15-CIZ, or one of the deletion mutants were stained with the anti-c-myc antibody and goat anti-mouse Alexa fluor 488 as secondary antibody. Slides were analysed with confocal microscopy (or fluorescense microscopy Q-T). **A**: CIZ; **B**: TAF15-CIZ; **C**: VP16-CIZ; **D**: E2A-CIZ; **E**: CIZ del NLS; **F**: CIZ del NLS, HEK293T; **G**: CIZ del ZF; **H**: CIZ del ZF, HEK293T; **I**: TAF15-CIZ del LZ; **J**: TAF15-CIZ del SR; **K**: TAF15-CIZ del PR; **L**: TAF15-CIZ del QA; **M**: TAF15-CIZ del NLS; **N**: TAF15-CIZ del NLS, HEK293T; **O**: TAF15-CIZ del ZF; **P**: TAF15-CIZ del ZF, HEK293T; **Q**,**R**: TAF15-CIZ del NLS, mitosis HEK293T; **S**,**T**: TAF15-CIZ del ZF, mitosis HEK293T; R and T represent DAPI staining.

microscopy, immunofluorescence staining for all deletion mutants with exception of CIZ and TAF15-CIZ without the zinc fingers was exclusively nuclear, spared nucleoli and associated with a finely granular distribution, frequently observed for RNA- and DNA-binding proteins [Spector, 1993]. Fluorescence was most intense within circular 'speckles' or domains distributed randomly within nuclei, against a less intense background of diffuse nuclear fluorescence (Fig. 6I–L). The nuclear pattern was not affected by deletion of the putative nuclear localisation signal, confirming that other sequences account for this (Fig. 6E,F, and M,N). In this respect, deletion of the zinc fingers resulted in a nuclear as well as a diffuse cytoplasmic staining in both cell lines (Fig. 6G,H, and O,P), confirming previous results [Feister et al., 2000]. In addition, staining of membrane ruffles was also detected. E2A-CIZ and VP16-CIZ exclusively accumulated in the nucleus with a comparable distribution as TAF15-CIZ (Fig. 6C,D). In general, expression of the CIZ deletion mutants was higher than the TAF15-CIZ fusions, but the expression pattern was similar between the corresponding deletion constructs and this in both cell lines.

Remarkably, staining of CIZ and TAF15-CIZ without the zinc fingers appeared to be excluded from condensed chromatin during mitosis

(Fig. 6S,T), whereas the speckled pattern of the other CIZ and TAF15-CIZ deletion mutants was greatly reduced and the signal was redistributed to condensed chromatin in mitotic cells (Fig. 6Q,R). Since CIZ is also known as a nuclear matrix protein and because of its possible interaction with chromatin, CIZ might link transcriptionally active chromatin to the nuclear matrix.

Transforming Properties of TAF15-CIZ Deletion Mutants

We then evaluated fibroblast transformation induced by the TAF15-CIZ deletion mutants. Transduction of NIH3T3 cells with either the empty vector or CIZ did not induce focus formation, whereas overexpressing TAF15-CIZ induced foci as described before. The TAF15-CIZ deletion mutant lacking the zinc fingers completely lost transforming capacity. In contrast, all other TAF15-CIZ fusions promoted foci with a biphenotypic morphology as observed for TAF15-CIZ (Fig. 7A). Moreover, these cells grew to a higher density when compared to cells overexpressing CIZ, except for the deletion mutant of the zinc fingers (Fig. 7B). Western blot analysis confirmed approximately equal expression of the recombinant proteins in the transduced cells (Fig. 7C). This implicates that differences in the transforming potential of the

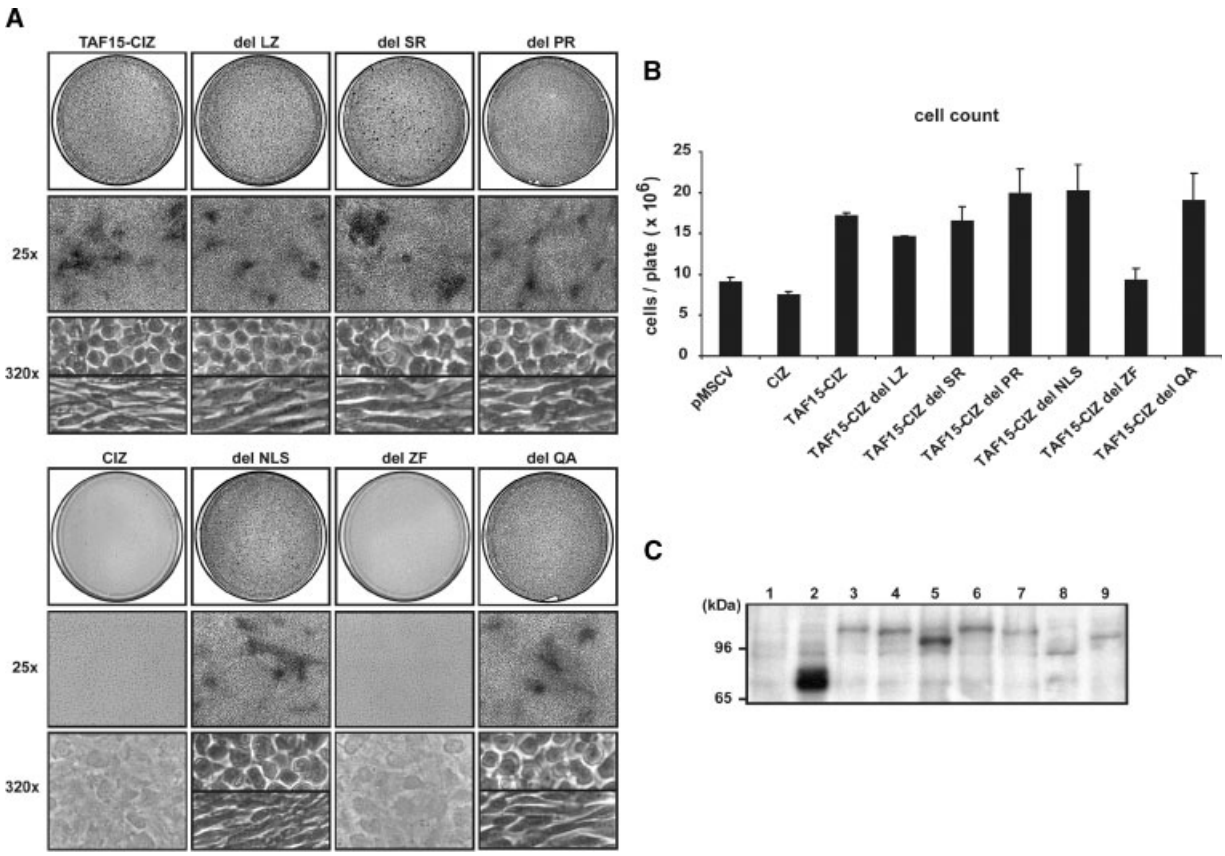

Fig. 7. Transforming properties of the TAF15-CIZ deletion mutants. **A**: Focus formation assay. NIH3T3 cells stably expressing one of the TAF15-CIZ deletion mutants were seeded at a density of 10^6 cells per 9 cm culture dish and cultured for 3 weeks. Macroscopic pictures were obtained after staining with 1% crystal violet in PBS. Microscopic pictures were taken at two different magnifications indicated at the left and illustrate at highest magnification two distinct populations: the spindle-like shape (lower) and the small round cell (upper) morphology. For TAF15-CIZ lacking the zinc fingers, a monolayer of cells was observed as seen with CIZ. **B**: Cell density. Cultures of cells overexpressing CIZ, TAF15-CIZ or a TAF15-CIZ deletion mutant were trypsinised at confluency after 3 weeks of culture and counted using a Coulter Multisizer. Both experiments were performed in duplicate, using independent transductions. **C**: Western blot analysis. Equal volumes of total cell lysate were separated on 10% SDS–PAGE and proteins were detected with anti-c-myc antibody. **Lane 1**, pMSCV-puro; **Lane 2**, CIZ; **Lane 3**, TAF15-CIZ; **Lane 4**, TAF15-CIZ del LZ; **Lane 5**, TAF15-CIZ del SR; **Lane 6**, TAF15-CIZ del PR; **Lane 7**, TAF15-CIZ del NLS; **Lane 8**, TAF15-CIZ del ZF; **Lane 9**, TAF15-CIZ del QA.

deletion mutants did not reflect alterations in protein expression levels, but resulted from differential function of the chimeric proteins. Thus, the zinc finger domain is essential for both DNA-binding and fibroblast transformation, whereas the other CIZ domains do not affect these properties.

DISCUSSION

In vitro studies on the transforming properties of TET-CIZ fusions showed that cells overexpressing either fusion do acquire a transformed phenotype in 3T3 fibroblasts (contact-independent growth) and display a morphological change. However, expression of TAF15-CIZ and EWSR1-CIZ could not induce substrate-independent growth in soft-agar assays, nor was there any difference in tumour-forming kinetics in nu/nu or SCID mice, suggesting that they have a weak transforming potential that is defined by the CIZ transcription factor. There is ample evidence showing that the oncogenic influence of fusion genes is dependent on cellular environments. Therefore, we tested the transforming properties of TAF15-CIZ and EWSR1-CIZ in more relevant haematopoietic cell culture systems as well. Expression of the TET-CIZ fusions in the IL3-dependent lymphoid Ba/F3 cells failed, however, to induce growth factor independence or increased resistance to apoptosis. In addition, the fusions were not able to inhibit differentiation into neutrophils of mouse myeloid precursor L-G cells and induce its G-CSF-dependent growth (data not shown). Therefore, we used the positive focus formation assay in 3T3 fibroblasts to study the structural requirements for oncogenic transformation by CIZ fusions in this system.

Our results reported here strongly suggest that aberrant transactivation is part of the molecular oncogenic mechanisms by CIZ fusions in 3T3 fibroblasts. First, the novel E2A-CIZ fusion as well as the artificial VP16-CIZ fusion is able to transform NIH3T3 cells. The presence of a transactivation domain derived from an RNA-binding protein is thus not strictly required for fibroblast transformation, albeit significant for the transformed phenotype. Second, deletion of the zinc fingers within TAF15-CIZ results in loss of both transforming and DNA-binding properties, indicating that DNA-binding is specifically required for transformation of 3T3 fibroblasts. Third, deletion of the zinc fingers in CIZ abolishes its transactivating properties.

Torrungruang and co-workers identified the transactivation domains within CIZ [Torrungruang et al., 2002a]. The N-terminus of CIZ exhibits a strong transactivating potential, but this activity is masked within the context of full-length CIZ. In addition, the poly(QA) domain at the C-terminus of CIZ also contains transactivating capacities that appear to depend on the nature of the DNA-binding domain. These observations suggest that CIZ is under allosteric control [Lefstin and Yamamoto, 1998], which is a significant mechanism for gene expression. These allosteric effects on CIZ may be very important for the action of the CIZ fusions as well. But since the MMPs are not significantly transactivated, this is probably not the case.

The transcription factor E2A contains two characterised activation domains (AD1 and AD2) [Aronheim et al., 1993], and is involved in ALLs with translocations t(1;19)(q23;p13) or t(17;19)(q22;p13), where both E2A activation domains are fused to the PBX1 homeodomain [Nourse et al., 1990] or the leucine zipper DNA-binding domain of HLF [Inaba et al., 1992], respectively. On the other hand, the E2A-CIZ fusion, produced by the translocation t(12;19)(p13;p13), comprises only the first activation domain AD1 of E2A fused to the full-length CIZ. In this respect, the transforming and transactivating efficiency of E2A-CIZ was less than that of VP16-CIZ, and is therefore consistent with earlier observations in which deletion of AD2 drastically impaired focus forming potential as well as transcriptional activation [Monica et al., 1994]. Furthermore, extensive analyses of E2A-PBX1 deletion mutants have demonstrated that both activation domains vary in their potencies in different cell types. Deletion of the AD1 domain abolished both fibroblast and lymphoid transforming activities, while elimination of the AD2 domain primarily reduced transformation in fibroblasts [Monica et al., 1994].

We already showed that TET-CIZ fusions did not significantly transactivate a reporter construct, carrying the CIZ consensus sequence, at the expression levels that can be experimentally obtained in HEK293T cells, although they could bind this specific sequence in EMSA experiments [Martini et al., 2002]. One possible explanation is that the expression levels of

TET-CIZ fusions were much lower than that of CIZ and could not be increased, possibly by their interference with proliferation of the cells, as already described for EWSR1-Fli1 [Deneen and Denny, 2001]. Alternatively, TET-CIZ fusions might require interaction with other co-factors absent in this system, and thus only function as effective transactivators in a different molecular context. It could also be that TET-CIZ fusions regulate a different set of target genes in comparison with CIZ, due to the protein–protein interactions that involve the transactivation domain. This does not diminish the importance of transactivation in transformation; rather it emphasises the relevance of cellular background and natural context for the activity of TET-CIZ chimera. Similarly, representational difference analyses performed on NIH3T3 fibroblasts overexpressing E2A-PBX1 identified a number of genes specifically activated by E2A-PBX1 in fibroblasts, but not in mouse myeloid cells transformed by E2A-PBX1, nor in fusion-positive human pre-B cell leukaemia lines [Fu and Kamps, 1997; McWhirter et al., 1997; Fu et al., 1999]. On the other hand, overexpression of CIZ led to a reproducible transactivating activity of *MMP7*, confirming earlier observations [Nakamoto et al., 2000; Torrungruang et al., 2002a]. However, the transactivating potential of CIZ was very low, when CIZ was expressed at similar levels as the TET-CIZ fusions. In this respect, it could be that the *MMPs* are not the key targets of CIZ and its fusions.

In conclusion, when CIZ is fused to a transactivation domain, it acquires transforming properties in 3T3 fibroblasts, which suggests that transactivation might be involved in transformation. In addition, our data suggest an essential role for the zinc fingers in DNA-binding and fibroblast transformation. Together, these observations support the hypothesis that the transforming activities of CIZ fusions might be correlated with their transactivating potential.

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