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CD13/APN transcription is regulated by the proto-oncogene c-Maf via an atypical response element

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Abstract

Angiogenic growth factors induce the transcription of the cell surface peptidase *CD13/APN* in activated endothelial cells of the tumor vasculature. Inhibition of *CD13/APN* abrogates endothelial invasion and morphogenesis *in vitro* and tumor growth *in vivo* suggesting a critical functional role for *CD13* in angiogenesis. Experiments to identify the transcription factors responsible for this regulation demonstrated that exogenous expression of the proto-oncogene c-Maf, but not other bZip family members tested, potently activates transcription from a critical regulatory region of the *CD13* proximal promoter between –115 and –70 bp which is highly conserved among mammalian species. Using promoter mutation, EMSA and ChIP analyses we established that both endogenous and recombinant c-Maf directly interact with an atypical Maf response element contained within this active promoter region via its basic DNA/leucine zipper domain. However full activity of c-Maf requires the amino-terminal transactivation domain, and site-directed mutation of putative phosphorylation sites within the transactivation domain (serines 15 and 70) shows that these sites behave in a dramatic cell type-specific manner. Therefore, this atypical response element predicts a broader range of c-Maf target genes than previously appreciated and thus impacts its regulation of multiple myeloma as well as endothelial cell function and angiogenesis.

Keywords

endothelial; aminopeptidase; angiogenesis; bZIP

1. Introduction

The *CD13* or aminopeptidase N (*CD13/APN*) cell surface glycoprotein is not expressed on resting vessels but its expression is induced on activated endothelial cells in response to angiogenic growth factors (Pasqualini et al., 2000; Bhagwat et al., 2001). *In vitro* inhibition of *CD13* with peptidase inhibitors or antibodies abrogates endothelial invasion and morphogenesis (Bhagwat et al., 2001; Bhagwat et al., 2003; Petrovic et al., 2003) and *in vivo* inhibition reduces angiogenesis and halts tumor growth (Pasqualini et al., 2000). Investigation into the pathways regulating the expression of *CD13* in activated endothelium may identify novel factors and fundamental processes involved in the activation of endothelial cells.

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Two distinct promoters regulate the transcriptional expression of *CD13* in a tissue specific manner (Shapiro et al., 1991). *CD13* expression in cells committed to the myeloid lineage is regulated by its distal promoter located eight kilobases upstream of the translational start codon, while transcription in endothelial cells and epithelial cells is directed by the proximal promoter immediately upstream of the start codon (Bhagwat et al., 2001). Through Ras-dependent mitogen-activated pathway kinase (MAPK) and phosphatidylinositol-3 kinase signaling pathways, angiogenic factor such as bFGF and VEGF activate the transcription factor Ets-2-directed transcription of *CD13* in endothelial cells (Bhagwat et al., 2001; Bhagwat et al., 2003; Petrovic et al., 2003). However, further experiments indicated that additional transcriptional mechanisms independent Ets-2 controlled *CD13* expression. In the current study we sought to identify auxiliary transcription factors that contribute to the regulation of *CD13* expression in endothelial cells.

Similar to the Ets family of transcription factors, the role of the AP-1 transcription factor is well established in vascular biology (Lelievre et al., 2001). The AP-1 factor is a heterodimeric complex consisting of members of either the Jun, Fos, ATF or Maf families of transcription factors (Eferl and Wagner, 2003). While the roles of Jun and Fos are established in vascular biology, the structurally related Maf proteins have not been studied in angiogenesis. In this investigation we establish the proto-oncogene c-Maf as a positive regulator of the *CD13* proximal promoter by its direct binding to a region highly conserved across mammalian species. Furthermore we demonstrate that the activity of c-Maf requires its transactivation domain where it is differentially regulated by two conserved serine residues. Our work suggests that not only can c-Maf activate transcription of its target genes via a novel response element, but that c-Maf may also regulate *CD13* in activated endothelial cells, identifying it as a potential therapeutic target in pathologic angiogenesis.

2. Materials and methods

2.1 Cell Culture and Transfections

Kaposi sarcoma derived endothelial (Ogawa et al., 2000., Herndier et al., 1994) and mouse hemangioendothelioma cell lines (Obeso et al., 1990) were maintained in Dulbecco modified Eagle medium (DMEM) under standard culture conditions. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics Corporation (San Diego, CA) and maintained according to the manufacturer's protocol.

Plasmids were transfected into EOMA cells as described (Petrovic et al., 2003) and luciferase values normalized to protein content. The siRNA were constructed with the Silencer siRNA Construction Kit (Ambion, Austin, TX) to target three different sequences within the coding region of c-Maf: 1179 Sense strand: aactccaggtgcgccttctgcctgtctc; 1184S: aagtagtcttcaggtgcgcc cctgtctc, 1996S: aattgtcgtctcgcagccgtcctgtctc (Hurt et al., 2004). The siRNA were tested for their specificity and efficiency of knocking down protein expression of newly transcribed RNA by co-transfecting various expression plasmids (c-Maf, MafB, and c-Fos) and 10nM of each individual siRNA.

2.2. Details of reporter and expression plasmids

The 5' deleted 153-bp reporter plasmid (153/*luc*) was previously characterized (Petrovic et al., 2003). The 115-bp reporter (115/*luc*) and the 70-bp reporter plasmid (70/*luc*) were constructed using PCR and incorporating a 5' XmaI and 3' HindIII site for cloning into pGL2 basic. Deletion of the c-Maf response element in the 153-bp reporter plasmid (Δ 120-87/*luc*) was made by incorporation of a KpnI site into the region by PCR mutagenesis. Site-directed mutation of motifs (113mut/*luc*, 104mut/*luc*, and 97mut/*luc*) were made using PCR to incorporate XmaI and HindIII sites for insertion into pGL2 vector. The *IL-4* (M1) promoter (−797) plasmid was

provided by Dr. M. Brown of Northwestern University (Tara et al., 1993). The -305/+5 *IL-4* promoter region was subcloned into pGL2 basic as described above with primers also used in the chromatin immunoprecipitation assay for the positive control: (IL-4.-302F 5'-tccccccggggaggagagccagtggaacc; IL-4. +5.R 5'-atgccaaagcttatgcaatgctggcagagatc). MafB, c-Maf and c-Maf mutant (C-term, PLER, R22E and L2P4P) expression plasmids were a gift of Dr. C. Kurschner; p18/MafK plasmid from Dr. P. Ney; c-Fos plasmid from Dr. T. Curran. The cJun plasmid was purchased from ATCC. c-Maf^{S15A}, c-Maf^{S70A}, c-Maf^{S15A/S70A} mutants were made by PCR with internal primers incorporating the mutation and external primers with 5' MluI and 3' ApaI sites, cloned into the c-Maf/RcRSV plasmid and sequence confirmed.

2.3. GST-fusion protein purification and electrophoretic mobility shift assay (EMSA)

The GST-cMaf plasmid (Hegde et al., 1998) consists of GST fused 5' of the C-terminal domain of c-Maf (aa 257–360) and contains the bZip domain essential for dimerization and DNA binding. Recombinant c-Maf was produced as previously described (Hegde et al., 1998) and proper binding was assayed by EMSA with Maf response elements (Kataoka et al., 1994). Gel shift assay reactions were performed with 1–2 µg of nuclear lysate or 0.5 µg purified c-Maf and 5,000–25,000 cpm probe as previously described (Hegde et al., 1998).

2.4. RT-PCR analysis and Southern blot hybridization

Total RNA was isolated using RNeasyStat (Iso-Stat Diagnostics, Inc., Friendswood, TX). cDNA was reverse transcribed with SuperscriptII (Invitrogen) and amplified with the forward (5'-gcttc gagaaaacggctc-3') and reverse (5'-tgccag tgggctcagttatg-3') primers as described (Hurt et al., 2004). PCR products were probed with the internal oligo 5'-cgacaacccgtcctctcc cgagttt-3' probe by Southern blot.

2.5. Western blot analysis

Whole cells were scraped directly into Laemmli buffer (Bio-Rad, Hercules, CA) and β-mercaptoethanol. Nuclear lysates were prepared as described (Schreiber et al., 1989). Antibodies (c-Maf, M153 and N15; v-Maf, N15; MafB, P20; MafF/G/K, C-18; c-Jun, D; and c-Fos, K-25) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). To immunoprecipitate c-Maf, 500 µg of KS1767 nuclear lysate was precleared and incubated with 10 µg of M153 c-Maf rabbit antibody (or reagent grade IgG), immunoprecipitated as specified by the manufacturer and immunoblotted with N14 c-Maf goat antibody.

2.6. Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed following the Upstate ChIP Assay Kit (–295, Upstate Cell Signaling Solution, a Serological Company) protocol with an additional step of nuclear preparation before sonication (Cao et al., 2005; Weinmann et al., 2001). Nuclei were resuspended in lysis buffer and sonicated with the Dismembrator to produce chromatin fragments between 300 and 500-bp. Immunoprecipitation was performed with the anti-c-Maf (M153) antibody and twenty percent of the supernatant from the rabbit IgG immunoprecipitation was kept as a PCR positive control as the 'input'. ChIP samples were eluted from agarose beads, ethanol precipitated and resuspended in 50 µl water. PCR analysis (conditions 95°C, 5min; 95°C, 30s; 60°C, 45s; 72°C, 45s; for cycles indicated per primer pair; 72°C, 10 minutes) was performed on 5 µl sample with primers specific for the proximal promoter of CD13 (153.F: agtccagtgacctcgctgttgagccctggttaatt; 33R: ttggggaggcgg ctgaggagcctcaggcca); as a negative control, for the distal promoter of CD13 (411F: tctagactacgagtcagggtacc; 130R: gtgtcccgaggc agcctgtg) and as a positive control, for IL-4 promoter (primers described in section 2.2). Relative intensities were calculated with the Kodak Molecular Imaging Software (Eastman Kodak Company, Rochester, NY).

3. Results

3.1. The region between –153 and –70 of the proximal promoter is essential for activity in endothelial cells

To identify essential *cis*-acting elements regulating *CD13* transcription in endothelial cells, we assayed the transcriptional activity of a series of reporter plasmids containing progressive 5' deletions of the one-kilobase proximal promoter which we have previously shown to control endothelial *CD13* transcription [ref. (Bhagwat et al., 2001) Fig. 1A]. We used the murine hemangioendothelioma cell line (EOMA) as our model system which faithfully recapitulates *CD13* regulation in primary endothelial cells (Bhagwat et al., 2001, Bhagwat et al., 2003, Petrovic et al., 2003). While the –153bp promoter fragment (153/*luc*) was marginally less active (81%) than the full-length promoter (1kb/*luc*), further deletion of the sequences upstream of either –115bp (115/*luc*) or –70bp (70/*luc*) significantly compromised promoter activity (29% and 3% respectively). These experiments distinguished what appears to be two essential transcriptional regions; one located in a 38-nucleotide region between –153 and –115 bp; and the second located between –114 and –70 bp upstream of the start codon. Sequence alignment shows that the region between –153 and –70 is highly conserved across a number of mammalian species (Fig. 1B). This region has also been shown to contain multiple functional *cis*-acting elements that regulate *CD13* transcription in the porcine liver and intestine (Olsen et al., 1991). We have previously established that in endothelial cells, Ets-2 transactivates *CD13* through an element located in the region between –153 and –115bp (Petrovic et al., 2003). However, based on our deletion assays, Ets-2 is clearly not solely responsible for the *CD13* transcriptional activity in endothelial cells.

3.2. The bZip transcription factor c-Maf transactivates the CD13 promoter in endothelial cells

The bZip transcription factors, particularly AP-1, play important roles in angiogenesis and cellular differentiation (Blank and Andrews, 1997). To test whether *CD13* may be a bZip target, we co-transfected endothelial cells with a panel of expression plasmids containing individual bZip family members with the *CD13* 1kb/*luc* reporter plasmid and assayed for luciferase activity. Exogenous expression of c-Jun, c-Fos, MafB, or MafK produced negligible effects on *CD13*-driven luciferase activity as did cotransfection of c-Fos and c-Jun. However, exogenous expression of c-Maf activated 1kb/*luc* reporter gene transcription in a dose-dependent manner five to fifteen fold over values obtained in cells transfected with the expression vector alone (Fig. 2A). Furthermore transactivation by c-Maf expression plasmids was specifically abrogated by treatment of cells with siRNAs that effectively knock down newly transcribed c-Maf protein (Fig. 2B), confirming that transactivation depends on c-Maf (Fig. 2C).

3.3. c-Maf directly binds the –153 bp promoter region

Transcription factors are composed of discrete functional domains performing specific tasks that contribute to the overall activity of the molecule (Fig. 3A). To determine the functional domains of the c-Maf protein that are essential for maximal activation of the *CD13* promoter, we tested the ability of a panel of mutant c-Maf expression plasmids to transactivate the full length 1kb/*luc* in endothelial cells (Fig. 3B). Deletion of the N terminal three-fourths of the c-Maf cDNA results in a protein lacking a transactivation domain but retaining the domains essential for both protein dimerization and DNA binding [C-term plasmid, aa 257–340 (Kurschner and Morgan, 1995)]. Co-transfection of this plasmid with the 1kb/*luc* reporter impaired but did not completely abrogate c-Maf-directed transcription (Fig. 3B), suggesting that the transactivation domain is essential for maximal activity. However, the failure to completely arrest transcription may reflect the fact that this truncated protein retains its ability to heterodimerize with endogenous partners via its intact leucine zipper and thus may recruit transcriptional coactivators to the promoter. In contrast, disruption of bZip domain-dependent

protein-protein interactions by substitution of two of the six leucine residues in the zipper region with proline resulted in complete abrogation of the ability of c-Maf to induce *CD13* promoter activity in agreement with the requirement for bZip domain-dependent protein-protein interactions [L2P4P mutant (Kataoka et al., 1993), Fig 3B]. Finally, co-transfection of expression plasmids containing mutations disturbing c-Maf binding to DNA again showed impaired transactivation when compared to the intact protein [PLER (Kurschner and Morgan, 1995) and R22E (Kataoka et al., 1993), Fig 3B] with the more extensive alterations of PLER resulting in a more profound effect on c-Maf's transactivation potential, suggesting that c-Maf may directly bind the promoter through this domain.

To determine if c-Maf transactivates through one of the transcriptionally relevant regions identified by our deletion analysis, we co-transfected the c-Maf expression plasmid with the $-153/luc$, $-115/luc$, and $-70/luc$ reporter constructs. While deletion of the region between -153 and -115 did not have a significant effect on the ability of c-Maf to transactivate the reporter constructs, elimination of the second region between -115 and -70 significantly reduced transactivation (Figure 3C), implying that c-Maf acts via a motif contained within this promoter region. To confirm a direct interaction between c-Maf and the promoter, we used the recombinant bZip domain of the c-Maf protein (Hegde et al., 1998) in an electrophoretic mobility shift assay (EMSA, Fig. 3D). We determined that c-Maf is able to bind the 153bp promoter region which is inhibited by addition of excess unlabeled oligonucleotide containing the Maf recognition element (Fig. 3D, lane 3). In contrast, competition with an oligonucleotide spanning the region between -153 to -115 containing the Ets-2 motif (Petrovic et al., 2003) was not observed (Fig. 3D, lane 4).

3.4. c-Maf acts through an atypical response element

Because c-Maf can directly bind the *CD13* promoter and the relevant region of the *CD13* promoter does not contain a canonical palindromic Maf response element, we initiated an unbiased assessment of the putative Maf binding site. Competition analysis using a series of overlapping unlabelled oligonucleotides spanning the relevant promoter region showed that only the oligomer analogous to the -120 to -87 region was able to abrogate binding of the recombinant c-Maf to the -153 to -1 promoter fragment probe (Fig. 4A). Partial inhibition shown with sequences overlapping and immediately upstream of this region (-132 to -107) suggests that the c-Maf recognition site may extend to these sequences as well. In agreement with this notion, EMSA using the -120 to -87 oligomer as a probe indicated that purified c-Maf binds to these sequences, albeit with lower efficiency than the 153bp probe (data not shown).

While consensus Maf recognition elements have been identified (Kataoka et al., 1994), further investigation has shown that the binding of Maf proteins to DNA is quite complex. Analysis of many other c-Maf responsive promoters indicates that the TGC nucleotides that flank the classical AP-1 response element (TGC_n₆₋₇GCA) actually specify Maf binding [Fig. 4B, (Dlakic et al., 2001)]. Inspection of the c-Maf binding sequence in *CD13* showed that it contains two TGC motifs between -120 and -87 bp. To assess the functional importance of these sequences to *CD13* promoter activity, each of the TGC motifs were altered via site-directed mutagenesis within the context of the *153/luc* plasmid (Fig. 4C). Mutation of the $^{-113}\text{TGC}^{-110}$ motif significantly abrogated transcription from the *CD13* promoter in endothelial cells (Fig. 4D) while the other two mutations had a negligible effect on activity. Interestingly, sequences immediately upstream of this functional TGC motif are extremely AT-rich, comparable to previously characterized Maf response elements [Table 1, (Yoshida et al., 2005)] and possibly indicating an extended c-Maf recognition site. Indeed, deletion of the -120 to -87 region in the context of the 153 bp promoter completely abrogated *CD13* promoter activity, supporting this concept (Fig. 4D).

3.5. Putative phosphorylation sites of c-Maf are differentially utilized

bZip transcription factor activity is controlled via phosphorylation of serine and/or threonine residues (Civil et al., 2002, Kawauchi et al., 1999). Sequence alignment of the large Maf proteins showed that numerous putative phosphorylation sites in the amino terminal transactivation domain are highly conserved (Civil et al., 2002) a region we find critical for maximal activation of the *CD13* promoter in endothelial cells. We chose to focus on two ERK phosphorylation sites which are strictly conserved in all of the large Maf proteins (serines 15 and 70 in the c-Maf sequence, Fig. 5A) and functionally regulate the related protein L-Maf, yet are uncharacterized in c-Maf (Civil et al., 2002, Kawauchi et al., 1999). We introduced mutations into the c-Maf expression plasmid to alter these serine residues (Ser¹⁵ and Ser⁷⁰, analogous to Ser¹⁴ and Ser⁶⁵ in L-Maf/MafA) to alanine residues, either individually or in combination and confirmed the expected expression profile by western blot analysis (Fig. 5B). The observed mobilities are comparable to those described for the analogous L-Maf mutants where proteins harboring the S65A mutation showed faster mobility than either wild type or those containing the S14A alteration (Benkhelifa et al., 2001) and are independent of the phosphorylation status of the protein.

Maf family members have been reported to act as both transcriptional activators or repressors and their characterized phosphorylation sites can serve to either stimulate or inhibit function (Nishizawa et al., 2003, Ochi et al., 2003). To determine if the c-Maf point mutants (S15A, S70A, and S15A/S70A) display cell type-dependent functional variability, we assessed their transactivation capacity in endothelial and liver epithelial cell lines (153/*luc*, Fig. 5C). We also assessed their activity in both cell lines using the *IL-4* c-Maf responsive promoter (−302/+5m1) to establish whether these mutants also display promoter-dependent functional variability (*IL-4/luc*, Figure 5D). While the c-Maf^{S70A} mutation did not significantly alter c-Maf induced *CD13* transcription in either cell line, it markedly increased liver specific transactivation of the *IL-4* promoter. The negative effect of the c-Maf^{S15A} mutation on the *CD13* promoter is only apparent in endothelial cells (Fig 5C left). These results demonstrate the context dependence of c-Maf transcriptional regulatory activity.

3.6. c-Maf is expressed in endothelial cells

To confirm that c-Maf is endogenously expressed in endothelial cells we amplified c-Maf RNA from human primary endothelial cells and human and murine endothelial cell lines by RT-PCR and confirmed the identity of amplified products by southern blot analysis using a c-Maf specific internal oligonucleotide probe. Primary human umbilical vein cells (HUVEC), the human Kaposi's sarcoma endothelial cell line KS1767 and the murine EOMA cell line all expressed detectable levels of c-Maf mRNA (Fig. 6A). The c-Maf overexpressing human multiple myeloma line OPM2 served as a positive control (Hurt et al., 2004).

To assay whether endogenous c-Maf interacts with the relevant *CD13* promoter region, nuclear extracts of KS1767 cells were incubated with the 153bp probe. Since KS1767 cells express high levels of endogenous CD13, they presumably contain *CD13* regulatory factors and thus are a useful model for assaying nuclear factors in activated endothelial cells (Bhagwat et al., 2003, Petrovic et al., 2003). Multiple complexes were found to bind the promoter region from the endothelial nuclear proteins in an electrophoretic mobility shift assay (EMSA, Fig. 6B). The binding of the slowest migrating complex to the probe behaved similarly to recombinant c-Maf: inhibition of complex formation by excess unlabeled oligonucleotide containing the Maf recognition element (Fig. 6B, lanes 2,4,7), which was unaffected by the oligonucleotide containing the Ets-2 motif (−153 to −115, Fig. 6B, lane 8), suggesting that this complex contains proteins in the Maf family. While the low level of endogenous c-Maf protein in endothelial cells was not demonstrable in nuclear lysates, it was evident upon immunoprecipitation followed by Western blot analysis (KS1767 cells, Fig. 6C). Therefore,

c-Maf mRNA and protein are expressed in CD13 positive endothelial cells. To assess if endogenous c-Maf proteins bind to the CD13 promoter within the nucleus, a chromatin immunoprecipitation assay was performed. DNA immunoprecipitated with antibodies directed against the c-Maf protein amplified with either PCR primer pairs specific for sequences -153 to -1 of the proximal promoter or the positive control IL-4 promoter were both enriched for their respective target chromatin, while the negative control primer pairs designed to detect a region of similar size in the distal promoter of CD13 did not (8 kb upstream, Figure 6D). This data supports that endogenous c-Maf interacts with the CD13 proximal promoter in endothelial cells, consistent with our hypothesis that c-Maf contributes to CD13 expression in endothelial cells.

4. Discussion

In the present study, we demonstrate that c-Maf activates transcription through an atypical motif in the *CD13* proximal promoter. The bZip transcription factors classically bind palindromic response elements as dimers. However the Maf transcription factors frequently activate endogenous promoters through response elements that consist of only one half of the canonical palindromic Maf response element (Yoshida et al., 2005). c-Maf induces *CD13* transcription by directly binding the minimal half site through its basic DNA/leucine zipper domain. This has been attributed to the presence of a unique extended homology region in the Maf bZIP subfamily that encodes a helical ancillary binding domain. This helical domain is thought to alter the conformation of the DNA binding region resulting in interactions distinct from those of c-Jun and other bZip dimers (Dlakic et al., 2001). In the present study, we show that the AT-rich region adjacent to the TGC motif in the *CD13* proximal promoter is required for c-Maf binding. Since it is becoming clear that Maf proteins can recognize and transactivate through motifs bearing minimal homology to the established consensus site, it is reasonable to suspect that the pool of Maf targets is considerably larger than that predicted by simple consensus site searches.

Aberrant expression of c-Maf activates multiple pathways that mediate tumorigenesis either by the direct induction of cell cycle proteins or indirectly by promoting tumor-stroma interactions (Hurt et al., 2004, Suzuki et al., 2005). While ectopic expression of c-Maf transforms chicken embryonic fibroblasts (Kataoka et al., 1993), overexpression of c-Maf, MafB, or L-Maf in primary embryonic neuroretinal cells does not result in their transformation. This finding highlights the significance of proper regulation of c-Maf expression. While the precise mechanisms regulating the functional activity of many of the Maf proteins are unclear, modification of the transactivation domain further regulates c-Maf activity, as evident by functional assay of deletion and site-directed mutations of c-Maf. Our finding that the c-Maf^{S15A} and c-Maf^{S70A} mutants behave in a distinct cell type specific fashion is interesting in comparison with L-Maf activation of the *crystallin* gene. We find that alteration of the Ser¹⁵ and Ser⁷⁰ residues increases c-Maf activity on the *CD13* promoter in the HepG2 liver cell line and thus are inhibitory. However others have found that the S14A/S65A double mutant of L-Maf significantly reduced L-Maf activity (Ochi et al., 2003, Benkhelifa et al., 2001). The mechanisms regulating activation of Maf family proteins have been investigated (Gardner and Montminy, 2005) and include a model invoking bFGF/MEK/ERK-induced phosphorylation that triggers proteasome-dependent degradation (Ochi et al., 2003) to explain L-Maf's decreased stability in neural retinal cells. In contrast, inhibition of the MAPK, MEK, increased c-Maf transactivation of the *gammaD crystallin* gene (Civil et al., 2002), while we observe that a similar MEK inhibition decreases c-Maf transactivation of the *CD13* promoter in endothelial cells to a similar extent as mutation of Ser¹⁵ (unpublished observations, Mahoney and Shapiro). Therefore, in endothelial cells the Ras/MAPK signal transduction pathway appears to activate c-Maf and *CD13* transcription (Bhagwat et al., 2003) but inhibits L-Maf (and c-Maf) activity

in primary neural retinal cells (Ochi et al., 2003). Thus its effects on Maf family members are particularly cell- and promoter-dependent.

Maf family members also frequently cooperate with members of other transcription factor families (Ho et al., 1996, Huang et al., 2002, Rajaram and Kerppola, 2004, Sevinsky et al., 2004, Aramata et al., 2005) and can also interact with transcriptional coactivators, such as CREB-binding protein and p300 to activate gene transcription (Chen et al., 2002). In our hands co-transfection of c-Maf with either Ets-2 or c-Jun expression plasmids did not show synergistic effects between these transcription factors on the *CD13* proximal promoter (data not shown). Investigations to identify additional proteins that cooperate with c-Maf in endothelial cells are ongoing on our laboratory. Since *CD13* is transcriptionally induced in tumor endothelium, further investigation into the role of c-Maf as an activator of angiogenesis is clinically relevant. We were unable to alter endogenous levels of *CD13* in our endothelial models, through either overexpression or knockdown of c-Maf. While we found effective and specific siRNAs for knocking down transfected c-Maf protein levels, experiments using these siRNAs to reduce endogenous levels of c-Maf were much less effective and produced only a 40–50% inhibition of c-Maf mRNA levels by RT-PCR after five days in culture. Not only does c-Maf likely function at low concentrations but the c-Maf protein may be quite stable and relatively refractory to siRNA effects.

c-Maf null mice have been produced and found to die *in utero* between embryonic day 15.5 and 18.5 (Kim et al., 1999), precluding the analysis of *CD13* expression in pathologic angiogenesis models. The cause of the premature death in the c-Maf null mouse has not been determined, but the relatively late stage mortality indicates that c-Maf is either compensated for by another large Maf or not involved in embryonic vasculogenesis. However, pathologic angiogenesis in the adult is believed to occur via processes distinct from those of normal developmental angiogenesis (Marchio et al., 2004, Marler et al., 2002, Becker et al., 2005) and thus formation of functional embryonic vasculature in the absence of c-Maf does not preclude its critical role in angiogenesis in the adult. An endothelial-specific c-Maf null animal model will be useful for addressing this question. Considering its function as an oncogene, c-Maf provides a particularly interesting transcriptional regulator in endothelial cells. The aberrant and specific upregulation of the expression of cyclin D2, integrin β 7, and the chemokine receptor CCR1 genes has been reported in c-Maf overexpressing multiple myeloma cell lines which contributes to tumorigenicity (Hurt et al., 2004). Since endothelial cells normally express these genes (Strasly et al., 2004) they may also be angiogenically relevant targets of c-Maf in endothelial cells. Transcriptional control of regulators of pathologic angiogenesis on activated endothelium is an important aspect of our understanding of those signals and proteins that contribute to the establishment of an actively angiogenic state. Further investigation of c-Maf regulated genes is essential for a greater understanding of its relevance to human disease.

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Abbreviations

CD13/APN

alanyl aminopeptidase, aminopeptidase N, microsomal aminopeptidase, gp150, Myeloid plasma membrane glycoprotein CD13

c-Maf

homolog of avian musculoaponeurotic fibrosarcoma AS42 viral oncogene

bZip

basic DNA/leucine zipper domain family of transcription factors

EMSA

electrophoretic mobility shift assay

HUVEC

human umbilical vein endothelial cells

PCR

polymerase chain reaction

GST

glutathione S-transferase

μg

microgram

Figure 2. c-Maf transactivates the *CD13* promoter in endothelial cells via a response element between -153 and -70

A. EOMA cells were transiently cotransfected with 0.5 μ g/well 1kb/*luc* reporter and increasing amounts of plasmids expressing bZip family members: c-Maf, c-Jun, c-Fos, MafB, and MafK. Maximum fold activation was extrapolated from experimental data using GraphPad Software. Whole cell lysates from cells transfected with the bZip expression plasmids were analyzed for protein expression by Western blot. **B.** c-Maf, MafB, and c-Fos expression plasmids were cotransfected with the panel of siRNAs and assayed for protein expression of newly transcribed mRNA to confirm the efficacy and specificity of c-Maf siRNA treatment. Membranes were stripped and reprobed with the GAPDH antibody as a loading control. **C.** The panel of siRNA was cotransfected with the c-Maf expression plasmid and the *CD13* proximal promoter reporter plasmid to confirm that *CD13* transactivation is c-Maf specific.

luc, 115/*luc*, and 70/*luc* reporter plasmids) were cotransfected with c-Maf expression plasmid into EOMA cells. Luciferase activity is normalized to μg protein and is represented as percent transactivation of values obtained with full length c-Maf. Error bars refer to the standard derivation within one experiment performed in triplicate. Each experiment was performed at least three times and a representative experiment is shown. **D.** EMSA with the recombinant GST fusion protein containing the bZip domain of c-Maf. Competitions were performed with excess oligonucleotide with either the MARE sequence (lane 3) or the sequence between -153 and -115 (lane 4).

Figure 4. c-Maf acts through an atypical response element within the proximal promoter of *CD13*

A. EMSA with recombinant c-Maf and 153bp probe. Competitions were performed with a series of oligonucleotides spanning the length of the 153bp fragment, as indicated above each lane. **B.** Unlike other bZip transcription factors which interact with the core TGACGTCA palindromic response element, Maf transcription factors interact with the flanking TGC motifs of the canonical MARE, as described (Dlakic et al., 2001). **C.** The sequence between -120 and -87 contains two TGC motifs (-113 and -104) and one TGT motif (-97). Site-directed mutations were introduced into the three separate motifs within the -120 to -87 region in the context of the 153bp/*luc* plasmid. **D.** Equal amounts of the reporter plasmids containing the mutations were transiently transfected into EOMA cells and harvested after 24 hrs. Luciferase

activity is normalized to μg protein and is represented as percent activation of the intact -153 bp promoter. Error bars refer to the standard deviation within one experiment performed in triplicate. The experiment was performed on at least three times and a representative experiment is shown.

Figure 5. c-Maf activity is dependent on two highly conserved c-Maf serine residues that are regulated differently in endothelial and liver cells

A. Two serine residues (S15 and S70 in c-Maf) within the amino-terminal transactivation domain are conserved across the large Maf family. **B.** Whole cell lysates from cells transfected with RcRSV, c-Maf or the c-Maf phosphorylation site mutants (S15A, S70A, S15A/S70A) were probed with the M153 c-Maf antibody and were stripped and probed with the GAPDH antibody, as a loading control. Arrows indicate mutation of the S70A within c-Maf alters the electrophoretic mobility in SDS-PAGE to a greater degree than the S15A mutation. **C.** The c-Maf and the c-Maf point mutant expression plasmids (S15A, S70A, S15A/S70A) were co-transfected with the 153/*luc* *CD13* reporter plasmid into endothelial (EOMA) or liver (HepG2) cell lines. In EOMA cells, the c-Maf^{S15A} had significantly less activity on the *CD13* promoter than c-Maf, while in HepG2 cells, c-Maf^{S70A} and c-Maf^{S15A/S70A} has significantly less activity than wild type c-Maf. **D.** The c-Maf and the c-Maf phosphorylation site mutant plasmids were co-transfected with the IL-4/*luc* reporter plasmid into EOMA or HepG2 cells. In HepG2 cells, the c-Maf^{S70A} and the c-Maf^{S15A/S70A} had significantly greater activity than c-Maf, while the difference seen between c-Maf^{S70A} and c-Maf^{S15A/S70A} was not significant (ns., $p=0.376$). Luciferase activity was normalized to μ g protein and is represented as fold transactivation over transient transfection with the empty expression vector RcRSV. Error bars refer to the standard derivation within one experiment performed in triplicate. The experiment was performed at least three times and a representative experiment is shown. An asterisk represents statistical significance versus the appropriate control in each group (* $p<0.05$, ns. not significant).

Figure 6. c-Maf is expressed in endothelial cells and directly binds the first 153bp of the endogenous proximal promoter of the *CD13* gene

A. Southern blot analysis of c-Maf after RT-PCR of total RNA from KS1767, EOMA, HUVEC or OPM2 multiple myeloma positive control cells. **B.** EMSA with nuclear lysates from KS1767 cells with the 153bp probe. Competitions were performed with excess oligonucleotide with either the canonical Maf response element (MARE) sequence (lanes 2, 4, 7) or the sequence between -153 and -115 (lane 8), as indicated above each lane. Arrows indicate the band specifically competed by the MARE oligonucleotide. **C.** Western blot analysis of immunoprecipitated c-Maf from KS1767 nuclear lysates. **D.** Chromatin immunoprecipitation from formaldehyde fixed KS1767 cells. Genomic DNA was PCR amplified with primers for the CD13 proximal promoter (27 cycles), the CD13 distal promoter (negative control, 28 cycles), and the IL-4 promoter (positive control, 26 cycles). Relative intensities of c-Maf immunoprecipitated PCR products were compared to rabbit IgG chromatin immunoprecipitated PCR products. Each experiment was performed at least three times and a representative experiment is shown.

Table 1

Alignment of multiple AT-rich Maf response elements in endogenous promoters [modified from(Yoshida et al., 2005)].

Gene:	Promoter response element:	Reference
Consensus	TGCTGAC(G)TCAGCA	(Kerppola and Curran, 1994)
Mouse α A-crystallin	TGTCTATGCTCAGCTCGGGGGAGA	
Mouse γ F-crystallin	CTTTTGCTGCTGTTTCCTGCCAACACA	(Liu et al., 1991)
Chicken α A-crystallin	CATTTCTGCTGACCACGTTGCCCTC	(Matsuo and Yasuda, 1992)
Chicken B1-crystallin	AGGATGTGATGACTGGGCGGCCGCA	(Roth et al., 1991)
Chicken δ 1-crystallin	TGTTGTTGCTCACCTACCATGGACA	(Funahashi et al., 1991)
Mouse Hoxa-3	CCAAACTGCTGACGCGACTCTCACC	(Manzanares et al., 1999)
Mouse Hoxb-3	CGTAACTGCTGACTTAGGGGGATGA	(Manzanares et al., 1997)
Mouse Hoxb-3	CAAATTGTCAGACACCTACATTCT	(Manzanares et al., 1997)
Mouse IL-4	TAAAGTTGCTGAAACCAAGGGAAAA	(Ho et al., 1996)
Human CD13	TAATTTTGTCCCAGTCTGCCTGTTGTGGGGCTC	
5'consensus	AAATGCTGACTTT	(Yoshida et al., 2005)