Protein Stability and Domain Topology Determine the Transcriptional Activity of the Mammalian Glial Cells Missing Homolog, GCMb*

(Received for publication, September 16, 1999, and in revised form, November 30, 1999)

Elisabeth E. Tuerk‡, Jörg Schreiber‡, and Michael Wegner§

From the Zentrum für Molekulare Neurobiologie, Universität Hamburg, Martinistrasse 52, D-20246 Hamburg, Germany

The glial cells missing (GCM) family of transcription factors consists of Drosophila GCM and the mammalian proteins GCMa and GCMb. They are expressed in a highly restricted manner during development and are known or assumed to be important regulators of developmental fate decisions. As the biochemical properties of GCMb have not been studied so far, we have undertaken a detailed structure-function analysis of the mouse GCMb (mGCMb) protein. DNA-binding specificity was very similar to that of other GCM proteins. Nevertheless, mGCMb was only a weak transcriptional activator in a number of different tissue culture systems. Interestingly, this was not due to an intrinsic absence of transactivation potential. In effect, we were able to identify two separate transactivation domains within mGCMb, one carboxyl-terminally adjacent to the DNA-binding domain and the second within the extreme carboxyl terminus. Activity of both transactivation domains was, however, modulated by an inhibitory region unique to mGCMb and located between the two transactivation domains. Furthermore, pulse-chase experiments proved that the mGCMb protein has a half-life approximately four times shorter than mGCMa. Introduction of the above mentioned inhibitory domain of mGCMb into mGCMa shortened the half-life of mGCMa to a value typical of mGCMb with a concomitant reduction in transactivation potential. Given the strong correlation between protein stability and transactivation potential, functional differences between the two mammalian GCM homologs are likely due to differences in stability with a single inhibitory region in mGCMb being involved in the reduction of both.

Many important transcriptional regulators of vertebrate development have been identified by their homology to Drosophila proteins. The functions of the vertebrate homologs are often similar to those of their Drosophila counterparts. While studying Drosophila mutants with nervous system defects, a gene was isolated that was transiently expressed in cells of the nervous system destined to become glia (1–3). Because flies in which this gene was inactivated showed a selective loss of all glial cells in the nervous system with the exception of midline glia, the gene was named glial cells missing (gcm). Detailed inspection of the phenotypes resulting from gene inactivation and transgenic overexpression revealed that GCM2 is in fact the earliest marker known for cells of glial fate and is directly involved in the choice of an uncommitted precursor in favor of a glial and against a neuronal fate.

Biochemical analyses later showed that GCM had all the characteristics of a transcription factor with an amino-terminal DNA-binding domain and a carboxyl-terminal transactivation domain (4, 5). At the time of its identification, GCM did not show any homology to proteins in the data bases. However, intense investigations have since led to the description of two mammalian proteins with sequence similarity to GCM (4–9). These proteins are named GCMa (or GCM1) and GCMb (or GCM2) with a single letter prefix usually indicating the species of origin. Drosophila GCM and its two mammalian relatives thus make up a novel family of transcription factors.

Sequence homology between GCM and the mammalian GCMa and GCMb is restricted to the amino-terminal region, which contains the DNA-binding domain. Sequence comparison between the DNA-binding domains of Drosophila GCM and mouse GCMa (mGCMa) has in fact helped us to define the important structural determinants of the DNA-binding domain. Site-directed mutagenesis revealed the importance of seven symmetrically spaced cysteine residues and an adjacent lysine residue for integrity and binding capability of the DNA-binding domain (10).

The functions of the mammalian GCM family members are not well understood at the moment. Under the assumption that conservation of sequence is a good indicator for conservation of function, one might expect these proteins to have a role in mammalian gliogenesis. Developmental in situ hybridization studies, however, have so far only revealed GCMa expression in the embryonic trophoderm (6, 11, 12) and GCMb expression in the forming parathyroid gland (9).

When mouse GCMa was expressed as a transgene in the developing nervous system of Drosophila, formation of surplus glial cells was observed (9, 13). Ectopic expression in the fly thus seems to indicate that GCMa has very similar functional characteristics as Drosophila GCM. In agreement, both proteins behaved virtually interchangeably in transiently transfected cells when assayed for transcriptional activity (13). Similar to GCM, GCMa also had a strong transferable transactivation domain in its 80 carboxyl-terminal amino acids.

GCMb on the other hand behaved very differently when expressed as a transgene throughout the developing nervous system of Drosophila. Transformation of presumptive neurons into glia was not observed despite the fact that all parameters were identical to the ones used for ectopic GCMa expression (9).

* This work was supported by Grant SFB 444 from the Deutsche Forschungsgemeinschaft (to M. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Contributed equally to this work.

§ To whom correspondence should be addressed. Tel.: 49 40 42803 6274; Fax: 49 40 42803 6602; E-mail: wegner@plexus.uke.uni-hamburg.de.

1 The abbreviations used are: GCM, glial cells missing; mGCMa, mouse GCMa; CMV, cytomegalovirus; DBD, DNA-binding domain.
Thus it was concluded that GCMb differs from GCMa and Drosophila GCM in its functional characteristics. To understand this difference in function and to obtain an idea of the structure of GCMb, we have undertaken a detailed comparative analysis of its properties in tissue culture.

MATERIALS AND METHODS

Plasmids—A fragment spanning the complete open reading frame of mGCMb (sequence similar to Ref. 9) was isolated from E15 mouse embryo cDNA and inserted into the mammalian pCMV5 and the insect pDS47/V5 expression vector. As previously reported for mGCMa (10), the amino-terminal 184 amino acids of mGCMb were amplified from this fragment by polymerase chain reaction and cloned between EcoRI and SalI sites of pCMV5 yielding the eukaryotic expression vector for the mGCMb DNA-binding domain. For detection, mGCMb constructs were also tagged with an amino-terminal T7 epitope (Novagen). Tagged and untagged versions behaved identically in all functional assays. To generate chimeras between mGCMa and mGCMb, fragments corresponding to the respective amino-terminal DNA-binding domains or all of the following sequences were generated by PCR and combined crosswise making use of an introduced SalI site before final insertion into pCMV5 (for schematic representation see Fig. 4A).

An expression plasmid for mGCMb with an amino-terminal T7 epitope was constructed in a manner analogous to mGCMb. Using a polymerase chain reaction-dependent approach, amino acids 300–345 were deleted from GCMa under simultaneous introduction of a BglII site, producing mGCMaΔ. Consecutively, the same BglII site was used to introduce amino acids 266–352 of mGCMa into mGCMaΔ, yielding mGCMaΔΔ. Both mGCMaΔ and mGCMaΔΔ were inserted into pCMV5 for eukaryotic expression. All other mGCMa expression plasmids and the luciferase reporter plasmid 6xgbs luc were as described (4, 13).

mGCMb and mGCMa DNA-binding domains of the Tst-1/Oct6/SCIP protein (14) were amplified by PCR using regular oligonucleotide primers. The PCR fragments were cloned into the expression vectors pCMVGal4 and pCMV-TPOU containing coding sequences for the DNA-binding POU domain of Tst-1/Oct6/SCIP, a purified mouse monoclonal antibody directed against the T7 tag epitope (Novagen), and a purified mouse monoclonal antibody directed against the Gal4 DNA-binding domain (CLONTECH). Primary antibodies were used at dilutions ranging from 1:3000 to 1:12,000 and reacted with a suitable horseradish peroxidase-coupled secondary antibody.

Electrophoretic mobility shift assays were performed as described (10) with whole cell extracts from transiently transfected COS cells. Briefly, 0.5 ng of 32P-labeled probe (sequences as shown in Fig. 2A) was incubated with extract for 20 min on ice in a 20-μl reaction mixture containing 10 mM Hepes, pH 7.5, 50 mM KCl, 5 mM MgCl2, 2 mM dithiothreitol, 0.1 mM EDTA, 2 μg of bovine serum albumin, and 1 μg of poly(dI-dC) as unspecific competitor. Samples were loaded onto native 4% polyacrylamide gels and electrophoresed at 4 °C in 0.5 × TBE (45 mM Tris, 45 mM borate acid, 1 mM EDTA, pH 8.3).

RESULTS

Comparison of the amino acid sequence of mGCMb with those of mGCMa and GCM reveals significant homology only in the amino-terminal DNA-binding domain. Within the DNA-binding domain of mGCMa, we have previously identified several amino acids, in particular seven symmetrically arranged cysteine residues and an amino-terminally adjacent lysine residue, which are essential for strength or specificity of DNA binding or influence the domain’s conformation (10). As shown in Fig. 1, all of these amino acids are present and conserved within the amino-terminal region of mGCMb leading to the assumption that mGCMb might have DNA-binding characteristics similar to mGCMa.

The preferred recognition element for Drosophila GCM had been determined to be 5′-ATGGGGGT-3′ (4, 5). We have previously used a set of oligonucleotide probes with this consensus sequence.
As mGCMb binds to the GCM consensus element with high affinity, it should be possible to analyze the function of mGCMb as a transcription factor in transiently transfected cells using luciferase reporters under the control of GCM consensus elements (4). mGCMb was expressed from its CMV-driven effector plasmid and was detected in its epitope-tagged version in COS cell nuclear extracts by Western blot analysis (Fig. 3A). mGCMa was detected using an antiserum directed against amino acids 169–436 of this protein (Fig. 3A) or in its epitope-tagged version by the same monoclonal antibody used for mGCMb (Fig. 3F).

When transient transfections were performed in U138 cells (Fig. 3B), mGCMb caused a 10-fold induction of a luciferase reporter with six tandemly arranged binding sites. In parallel transfections, mGCMa elicited a robust 180-fold stimulation of the same reporter. Thus, mGCMb is a relatively poor transcriptional activator in U138 cells when compared with mGCMa.

To analyze whether this effect was cell line-specific or a general phenomenon, we carried out additional transfections with the same luciferase reporter in a number of other cell lines. When COS cells were used, we obtained a 3-fold induction for mGCMb versus a 10-fold induction for mGCMa (Fig. 3C). In 293 cells, activation rates for mGCMb were higher, approximately 20–30-fold. Still, mGCMb was clearly less effective than mGCMa, which on average elicited a 100–110-fold stimulation in parallel transfections (Fig. 3, D). Thus, there is significant variation in the transcriptional capacity of mGCMb depending on the cell line used. These differences notwithstanding, mGCMb is always much less active than mGCMa.

As functional differences between both mammalian GCM homologs were originally observed in transgenic flies (9), we repeated our comparative transactivation studies in Drosophila S2 Schneider cells (Fig. 3E). Reporter gene activation achieved with mGCMb was again low. On average we obtained an 11-fold stimulation for mGCMb, compared with a 98-fold stimulation for mGCMa. Thus, mGCMb exhibits only 11% the activity of mGCMa in Drosophila cells. This low transcriptional activity could in fact explain the different behavior of mGCMa and mGCMb transgenes in Drosophila.

All transactivation studies reported so far were carried out under conditions of maximal reporter gene activation. Using increasing amounts of expression plasmids for the epitope-tagged versions of mGCMa and mGCMb, we next analyzed reporter gene activation in relation to steady-state levels of both proteins in transfected 293 cells. For any given amount of expression plasmid, activation rates were higher for mGCMa than for mGCMb (Fig. 3F). Even with the highest amount of mGCMb expression plasmid, reporter gene induction remained significantly below the activation rates obtained with as little as 50 ng of mGCMa expression plasmid.

Western blot analysis with an antibody directed against the epitope tag was used to study the steady-state levels of GCM proteins in the transfected 293 cells. Even though the same expression system was used for both proteins, the amount of accumulated mGCMb protein was significantly below the amount detected for mGCMa for any given amount of expression plasmid (Fig. 3G). Thus, there is a good correlation between the reporter gene activation achieved by each GCM protein and its ability to accumulate in the transfected cell.

To determine the region responsible for the lower transcriptional activity of mGCMb as compared with mGCMa, we generated chimeras between both proteins. Chimera C1 contained the DNA-binding domain of mGCMa in the context of mGCMb.
sequences. Chimera C2 carried the DNA-binding domain of mGCMb with all other sequences being derived from mGCMa (Fig. 4A). Transient transfections were performed with these chimeras in 293 cells, as these cells not only allowed a clear distinction between mGCMa and mGCMb transactivation levels but also showed mGCMb transactivation levels significantly above background. In these cells, chimera C1 exhibited a transactivation capacity similar to mGCMb (29-fold for C1 and 32-fold for mGCMb), despite the fact that the amino-terminal domain was mGCMa-derived (Fig. 4B). Chimera C2, on the other hand, was even more active than mGCMa (180-fold for C2 and 110-fold for mGCMa). Thus, transactivation of both chimeras does not correlate with the origin of the DNA-binding domain but instead with the origin of the sequences carboxyl-terminal to the DNA-binding domain. The latter sequences therefore have to be responsible for the functional difference between mGCMa and mGCMb.

In the case of mGCMa, the sequences following the DNA-binding domain contain two transactivation regions, one between amino acids 220 and 300 of mGCMa, the other from amino acid 349 to the carboxyl terminus (13). The presence of these transactivation domains is a good explanation for the strong transcriptional activity of mGCMa in transient transfection experiments. Both transactivation domains were transferable to the unrelated DNA-binding domain of the Gal4 transcription factor and functioned independently of each other.

To evaluate the presence of transactivation domains within those parts of mGCMb following its DNA-binding domain, we generated an in frame fusion of amino acids 174 to 504 to the Gal4 DNA-binding domain and assayed this protein for its ability to activate a reporter gene driven by a Gal4-dependent promoter (Fig. 5A). We failed to detect reporter gene activation independent of whether U138, COS or 293 cells were used (Fig. 5B, and data not shown). In parallel transfections, the comparable mGCMa construct (Gal4(aC) in Fig. 5B) showed the expected activation (13). However, when the expression of the Gal4/mGCMb fusion was checked by Western blot analysis with an antibody directed against the Gal4 DNA-binding domain, we likewise failed to detect protein of the correct size (Fig. 5C). Other fusions between the Gal4 DNA-binding domain and shorter regions of mGCMb also failed to be detected in Western blots, despite the fact that all constructions were repeatedly verified by DNA sequencing (Fig. 5C and data not shown). This argues that the Gal4/mGCMb fusions were highly unstable in the cells and that transactivation domains, if present, could not be detected by this method.

We reasoned that the stability of mGCMb fusions might vary with the domain to which mGCMb regions are fused. Thus, we undertook a second attempt by fusing amino acids 174 to 504 to the Gal4 DNA-binding domain and assayed this protein for its ability to activate a reporter gene driven by a Gal4-dependent promoter (Fig. 5A). We failed to detect reporter gene activation independent of whether U138, COS or 293 cells were used (Fig. 5B, and data not shown). In parallel transfections, the comparable mGCMa construct (Gal4(aC) in Fig. 5B) showed the expected activation (13). However, when the expression of the Gal4/mGCMb fusion was checked by Western blot analysis with an antibody directed against the Gal4 DNA-binding domain, we likewise failed to detect protein of the correct size (Fig. 5C). Other fusions between the Gal4 DNA-binding domain and shorter regions of mGCMb also failed to be detected in Western blots, despite the fact that all constructions were repeatedly verified by DNA sequencing (Fig. 5C and data not shown). This argues that the Gal4/mGCMb fusions were highly unstable in the cells and that transactivation domains, if present, could not be detected by this method.

We reasoned that the stability of mGCMb fusions might vary with the domain to which mGCMb regions are fused. Thus, we undertook a second attempt by fusing amino acids 174 to 504 to the Gal4 DNA-binding domain and assayed this protein for its ability to activate a reporter gene driven by a Gal4-dependent promoter (Fig. 5A). We failed to detect reporter gene activation independent of whether U138, COS or 293 cells were used (Fig. 5B, and data not shown). In parallel transfections, the comparable mGCMa construct (Gal4(aC) in Fig. 5B) showed the expected activation (13). However, when the expression of the Gal4/mGCMb fusion was checked by Western blot analysis with an antibody directed against the Gal4 DNA-binding domain, we likewise failed to detect protein of the correct size (Fig. 5C). Other fusions between the Gal4 DNA-binding domain and shorter regions of mGCMb also failed to be detected in Western blots, despite the fact that all constructions were repeatedly verified by DNA sequencing (Fig. 5C and data not shown). This argues that the Gal4/mGCMb fusions were highly unstable in the cells and that transactivation domains, if present, could not be detected by this method.

We reasoned that the stability of mGCMb fusions might vary with the domain to which mGCMb regions are fused. Thus, we undertook a second attempt by fusing amino acids 174 to 504 to the Gal4 DNA-binding domain and assayed this protein for its ability to activate a reporter gene driven by a Gal4-dependent promoter (Fig. 5A). We failed to detect reporter gene activation independent of whether U138, COS or 293 cells were used (Fig. 5B, and data not shown). In parallel transfections, the comparable mGCMa construct (Gal4(aC) in Fig. 5B) showed the expected activation (13). However, when the expression of the Gal4/mGCMb fusion was checked by Western blot analysis with an antibody directed against the Gal4 DNA-binding domain, we likewise failed to detect protein of the correct size (Fig. 5C). Other fusions between the Gal4 DNA-binding domain and shorter regions of mGCMb also failed to be detected in Western blots, despite the fact that all constructions were repeatedly verified by DNA sequencing (Fig. 5C and data not shown). This argues that the Gal4/mGCMb fusions were highly unstable in the cells and that transactivation domains, if present, could not be detected by this method.
more stable than the analogous Gal4/mGCMb fusion.

Using a luciferase reporter which is activated by POU proteins, we analyzed this mGCMb fusion for its transactivation capacity (Fig. 6C). The POU domain of Tst-1/Oct6/SCIP did not lead to significant transactivation of the luciferase reporter on its own and served as a negative control. The Tst-1/Oct6/SCIP holoprotein, on the other hand, elicited a 7-fold stimulation of reporter gene expression. Similar activation rates were also observed for the mGCMb fusion indicating that there is indeed a transactivation domain between amino acids 174–504 of mGCMb.

To determine the borders of this domain, we generated further POU fusion proteins with mGCMb portions starting at amino acids successively closer to the protein's carboxyl terminus (Fig. 6C). The POU domain of Tst-1/Oct6/SCIP did not lead to significant transactivation of the luciferase reporter on its own and served as a negative control. The Tst-1/Oct6/SCIP holoprotein, on the other hand, elicited a 7-fold stimulation of reporter gene expression. Similar activation rates were also observed for the mGCMb fusion indicating that there is indeed a transactivation domain between amino acids 174–504 of mGCMb.

To determine the borders of this domain, we generated further POU fusion proteins with mGCMb portions starting at amino acids successively closer to the protein's carboxyl terminus (Fig. 6C). The POU domain of Tst-1/Oct6/SCIP did not lead to significant transactivation of the luciferase reporter on its own and served as a negative control. The Tst-1/Oct6/SCIP holoprotein, on the other hand, elicited a 7-fold stimulation of reporter gene expression. Similar activation rates were also observed for the mGCMb fusion indicating that there is indeed a transactivation domain between amino acids 174–504 of mGCMb.

To determine the borders of this domain, we generated further POU fusion proteins with mGCMb portions starting at amino acids successively closer to the protein's carboxyl terminus (Fig. 6C). The POU domain of Tst-1/Oct6/SCIP did not lead to significant transactivation of the luciferase reporter on its own and served as a negative control. The Tst-1/Oct6/SCIP holoprotein, on the other hand, elicited a 7-fold stimulation of reporter gene expression. Similar activation rates were also observed for the mGCMb fusion indicating that there is indeed a transactivation domain between amino acids 174–504 of mGCMb.

To determine the borders of this domain, we generated further POU fusion proteins with mGCMb portions starting at amino acids successively closer to the protein's carboxyl terminus (Fig. 6C). The POU domain of Tst-1/Oct6/SCIP did not lead to significant transactivation of the luciferase reporter on its own and served as a negative control. The Tst-1/Oct6/SCIP holoprotein, on the other hand, elicited a 7-fold stimulation of reporter gene expression. Similar activation rates were also observed for the mGCMb fusion indicating that there is indeed a transactivation domain between amino acids 174–504 of mGCMb.

To determine the borders of this domain, we generated further POU fusion proteins with mGCMb portions starting at amino acids successively closer to the protein's carboxyl terminus (Fig. 6C). The POU domain of Tst-1/Oct6/SCIP did not lead to significant transactivation of the luciferase reporter on its own and served as a negative control. The Tst-1/Oct6/SCIP holoprotein, on the other hand, elicited a 7-fold stimulation of reporter gene expression. Similar activation rates were also observed for the mGCMb fusion indicating that there is indeed a transactivation domain between amino acids 174–504 of mGCMb.

To determine the borders of this domain, we generated further POU fusion proteins with mGCMb portions starting at amino acids successively closer to the protein's carboxyl terminus (Fig. 6C). The POU domain of Tst-1/Oct6/SCIP did not lead to significant transactivation of the luciferase reporter on its own and served as a negative control. The Tst-1/Oct6/SCIP holoprotein, on the other hand, elicited a 7-fold stimulation of reporter gene expression. Similar activation rates were also observed for the mGCMb fusion indicating that there is indeed a transactivation domain between amino acids 174–504 of mGCMb.

To determine the borders of this domain, we generated further POU fusion proteins with mGCMb portions starting at amino acids successively closer to the protein's carboxyl terminus (Fig. 6C). The POU domain of Tst-1/Oct6/SCIP did not lead to significant transactivation of the luciferase reporter on its own and served as a negative control. The Tst-1/Oct6/SCIP holoprotein, on the other hand, elicited a 7-fold stimulation of reporter gene expression. Similar activation rates were also observed for the mGCMb fusion indicating that there is indeed a transactivation domain between amino acids 174–504 of mGCMb.

To determine the borders of this domain, we generated further POU fusion proteins with mGCMb portions starting at amino acids successively closer to the protein's carboxyl terminus (Fig. 6C). The POU domain of Tst-1/Oct6/SCIP did not lead to significant transactivation of the luciferase reporter on its own and served as a negative control. The Tst-1/Oct6/SCIP holoprotein, on the other hand, elicited a 7-fold stimulation of reporter gene expression. Similar activation rates were also observed for the mGCMb fusion indicating that there is indeed a transactivation domain between amino acids 174–504 of mGCMb.
parallel transfections the mGCMa fusion elicited activation rates comparable to the ones obtained with the corresponding mGCMb fusion (amino acids 426–504 in Fig. 6C).

A second deletion series of POU/mGCMb fusions was constructed with mGCMb fragments starting at amino acid 174 and extending variable distances toward the carboxyl terminus (Fig. 6A). A fusion that contained amino acids 174 to 353 of mGCMb exhibited a 13-fold induction of reporter gene expression compared with the 8-fold induction obtained with amino acids 174–504. A fusion with amino acids 174–263 of mGCMb yielded even higher induction rates as evident from the 29-fold stimulation in Fig. 6C. This result proves, on the other hand, that there are two completely nonoverlapping domains with transactivation potential in mGCMb. It confirms, on the other hand, the presence of an inhibitory domain contained within amino acids 266–352 of mGCMb. Thus, mGCMb has a structure very similar to mGCMa with regard to its transactivation domains. The transactivation domains per se do not seem to be weaker than the ones found in mGCMa. The main difference between the two mammalian GCM proteins is the inhibitory domain in mGCMb, which therefore might be responsible for the significantly weaker transcriptional activity of mGCMb as compared with mGCMa.

Taking our observation into account that mGCMb did not accumulate to the same amount in transfected cells as mGCMa and that fusions between portions of mGCMb and the Gal4 DNA-binding domain were extremely labile, we reasoned that part of a functional difference between both mammalian GCM homologs could also be based on differences in protein stability. To compare the stability of mGCMb and mGCMa, we performed pulse-chase experiments in transiently transfected COS cells (Fig. 7). This allowed us to follow specifically the decay of protein synthesized de novo during a 1-h period in the presence of 35S-labeled cysteine and 35S-labeled methionine over a 6-h chase period. When quantifying the amount of radioactivity present within mGCMb immunoprecipitated from such cells, it became evident that approximately two thirds of the protein had already disappeared after a chase period of 40 min (Fig. 7, A and B). After 2 h, only 17% of the radioactivity was still detectable and 95% of all label had disappeared from mGCMb immunoprecipitates after 6 h. Accordingly, the half-life of mGCMb was estimated to be approximately 30 min in these cells. When this experiment was repeated under identical conditions with mGCMa, we still retrieved almost half the label in immunoprecipitates after a chase period of 2 h (Fig. 7C). Even after 6 h, 20% of the total label remained detectable. The half-life of mGCMa was therefore estimated to be close to 2 h and is therefore in the same range as the one observed for Tst-1/Oct6/SCIP in transiently transfected COS cells (Fig. 7D). Thus, we conclude that mGCMb has a significantly shorter half-life and significantly higher turnover than mGCMa.

We also determined the half-lives of C1 and C2, the two chimeras between mGCMa and mGCMb previously analyzed for their transactivation potential. In pulse-chase experiments, chimera C1, which had transactivation characteristics similar to mGCMb, had a half-life of approximately 30 min (Fig. 7E). Chimera C2, on the other hand, exhibited a half-life of approximately 3 h (Fig. 7F), thus resembling mGCMa not only in terms of transcriptional activity but also in terms of stability. Both transactivation activity and stability of GCM proteins are determined by the region following the DNA-binding domain, suggesting a linkage between both properties.

Considering the importance of the previously identified inhibitory domain for the overall transcriptional activity of mGCMb, we were further interested to analyze its impact on protein stability. We removed a region corresponding to amino acids 266–352 from mGCMb and inserted it between the two transactivation domains of mGCMa (mGCMaΔb in Fig. 8A). The mGCMa protein carrying the inhibitory domain of mGCMb was detectable by Western blot analysis, just as the mGCMa protein in which the spacer region between both transactivation domains had been removed prior to insertion of the inhibitory domain of mGCMb.

![Image](https://example.com/image.png)

**Fig. 6. Transactivation domains in mGCMb.** A, summary of POU domain constructs. The mGCMb regions present in each POU domain fusion protein are indicated by the first and last amino acid still contained within the fragment. Tst-1, full length Tst-1/Oct6/SCIP, POU, POU domain of Tst-1/Oct6/SCIP, POU(ΔTA2) represents a fusion between the POU domain of Tst-1/Oct6/SCIP and amino acids 330–436 of mGCMa. B, expression of fusion proteins between the POU domain and mGCMb regions was analyzed by Western blots of nuclear extracts from transfected 293 cells with a polyclonal antiserum against the POU domain of Tst-1/Oct6/SCIP. Numbers indicate size of molecular mass markers in kDa. C, the POU-responsive luciferase reporter (HSV oct luc) was transfected into 293 cells together with expression plasmids for the isolated POU domain, together with expression plasmids for the POU domain of Tst-1/Oct6/SCIP, for full-length Tst-1/Oct6/SCIP, or for the POU domain fusions depicted in A. Luciferase activities were determined in three independent experiments, each performed in duplicates. Data are presented for each POU domain fusion as fold induction above the level of luciferase activity obtained in transfections with an expression plasmid for the isolated POU domain, which was given an arbitrary value of 1.
When analyzed for their ability to activate a GCM-dependent luciferase reporter in transiently transfected 293 cells, mGCMa without the spacer region stimulated expression of the reporter as efficiently as the mGCMa holoprotein (Fig. 8C). Insertion of the inhibitory domain of mGCMb, however, led to a dramatic loss of reporter gene stimulation, reducing induction rates to levels comparable to those of mGCMb and corresponding to approximately 25% of those obtained with wild-type mGCMa. Insertion of amino acids 266–352 of mGCMb also reduced the half-life of mGCMa as measured in pulse-chase experiments from approximately 2 h to less than 30 min (Fig. 8D). Thus, the inhibitory domain of mGCMb influences both protein turnover and transcriptional activity further supporting the close link between both properties.

DISCUSSION

The GCM family of proteins is a small, novel class of transcription factors consisting of Drosophila GCM and its two mammalian homologs mGCMa and mGCMb (5). Whereas GCM has a clear role in Drosophila gliogenesis (1–3), the function of its mammalian counterparts has yet to be defined. During embryogenesis, mGCMa is primarily expressed in trophoblasts of the placenta (11, 12). mGCMb, on the other hand, was selectively detected in the parathyroid anlage (9), arguing that it might play a decisive role in the generation of this gland. The expression patterns therefore seem to argue against an evolutionary conservation of function within the GCM family. This conclusion is somewhat unexpected from studies on Drosophila GCM. Here, ectopic expression of GCM was not only sufficient within the nervous system to transform cells into glia but even outside it in mesodermal and epidermal tissues, arguing that GCM is not dramatically dependent on a particular cellular environment to exert its function (18, 19).

Additionally arguing for a conservation of function, overexpression of mGCMa in the developing nervous system of Drosophila GCM. Here, ectopic expression of GCM was not only sufficient within the nervous system to transform cells into glia but even outside it in mesodermal and epidermal tissues, arguing that GCM is not dramatically dependent on a particular cellular environment to exert its function (18, 19).
Contrary to GCM and mGCMa, mGCMb has so far not been characterized biochemically. This study now fills the gap and provides several important insights into the biochemical properties of mGCMb. For one, we show that the DNA-binding characteristics of mGCMb are indeed very similar to those of the other GCM proteins. This is not only evident from the mobility shift experiments with variants of the GCM consensus binding site, but also from the strong transcriptional activation of promoters with GCM consensus binding sites by a chimeric protein that contained the DNA-binding domain of mGCMb. In agreement, all the amino acids previously identified as being important for mGCMa binding are fully conserved within mGCMb.

Despite this overall similarity, minor differences in the affinity toward GCM binding sites carrying particular mismatches were observed. This allows differential binding of mammalian GCM proteins to nonconsensus GCM binding sites and is therefore potentially relevant. The structural basis of these minor differences, however, cannot be determined without the availability of a crystal structure for a GCM-type DNA-binding domain and the possibility of comparative modeling.

Ectopic expression studies in transgenic flies had previously indicated that mGCMb was different from the other two GCM proteins because it did not cause the typical neuron-glia transformation (9). The failure of mGCMb to transform presumptive neurons into glia is unlikely to be due to differences in DNA binding. However, we reproducibly observed in a number of different cell lines that mGCMb was a significantly weaker transcriptional activator than either mGCMa or GCM. The amount of transcriptional activation obtained for mGCMb in different cell lines ranged from 5% to approximately 30% of the levels obtained with mGCMa. Importantly, mGCMb was only weakly active in Drosophila S2 Schneider cells, arguing that mGCMb might miss such strong transcription activation domains.

Using chimeras between mGCMa and mGCMb, we were also able to show that the difference in transcriptional potential is caused by those parts that follow the DNA-binding domain. These regions contain powerful transcriptional domains, both in mGCMa and in Drosophila GCM (4, 13). Thus it was reasonable to assume that the corresponding sequence of mGCMb might miss such strong transcriptional domains.

However, this was not the case. Fusions between various regions of mGCMb and the transcriptionally inactive POU domain of Tst-1/Oct6/SCIP clearly showed that transcriptional domains are present within mGCMb. In effect, these transcriptional domains were found at similar positions to those previously mapped in mGCMa and GCM, thus further corroborating the strong conservation of topology between members of the GCM family of transcription factors.

Comparison of these transcriptional domains with those of Tst-1/Oct6/SCIP and mGCMa showed that, at least in the context of POU domain fusions, they were not significantly weaker. Thus, we have to assume that the transcriptional domains per se cannot be the underlying reason for the weak transcriptional activity of mGCMb. mGCMb was, however, unique in possessing a strong inhibitory domain located between amino acids 258 and 347 (Fig. 9). The activity of the adjacent transcriptional domains was severely reduced in the presence of this domain. A region with comparable function had not been detected in mGCMa (13).

It was previously noted from sequence inspection that there are PEST sequences within mGCMb (7, 8), which have the potential to mark the protein for rapid degradation (21, 22). In total, there are three potential PEST sequences spread over wide parts of mGCMb that follow the DNA-binding domain (amino acids 204–236, 270–306, and 409–437). mGCMa contains only one such sequence (amino acids 239–261). Interestingly, the longest of the three PEST sequences in mGCMb is located within the region identified as a transcriptional inhibitory domain in this study (Fig. 9).

That protein stability could be an important determinant in explaining the functional differences between mGCMb and the other GCM proteins was indicated from two observations. First, mGCMb never accumulated to the same amount as mGCMa in transfected cells, and with equal amounts of expression plasmids there was always less mGCMb protein than mGCMa. Secondly, we failed to detect fusion proteins between the DNA-binding domain of Gal4 and mGCMb sequences. Why analogous fusions with the POU domain of Tst-1/Oct6/SCIP were detectable was not analyzed in detail. However, it seems reasonable to assume that the POU domain has a stabilizing influence on mGCMb regions.

Confirming the role of protein stability for transcriptional activity, we were able to show directly by pulse-chase experiments that in the same cellular environment mGCMb is much less stable than mGCMa. The half-life detected for mGCMb was approximately 30 min, whereas the half-life of mGCMa was close to 2 h and therefore not significantly different from that of the POU protein Tst-1/Oct6/SCIP. After 6 h, almost all of the mGCMb protein synthesized during the pulse period had disappeared, whereas 20–30% were still present for mGCMa and Tst-1/Oct6/SCIP, respectively. Thus, mGCMb turns over with a half-life comparable to c-Myc which is known to be extremely labile (23). For comparison, many other transcription factors are significantly more stable with reported half-
lifes ranging from 2 to 9 h (24–26). Turnover characteristics of the mGCMb protein were determined by the region carboxyl terminally adjacent to its DNA-binding domain as evident from the half-lives of chimeras between mGCMa and mGCMb. A chimera with the DNA-binding domain of mGCMa and the carboxyl-terminal sequences of mGCMb had turnover and transactivation potential similar to mGCMb. Intriguingly, insertion of a region corresponding to amino acids 266 to 352 of mGCMb into mGCMa was sufficient to reduce both transcriptional activity and half-life of mGCMa to levels typical of mGCMb, indicating the importance of this region in determining the biochemical properties of mGCMb. As already mentioned, this region contains one of the three PEST sequences identified by sequence inspection in mGCMb. It is tempting to speculate that this region marks mGCMb for rapid degradation through its PEST sequence and thereby determines the low transcriptional activity of this protein. Functional differences between mGCMa and mGCMb thus are primarily due to differences in protein stability, and not so much due to different DNA-binding specificities or different strengths in the respective transactivation domains.

REFERENCES
1. Hosoya, T., Takizawa, K., Nitta, K., and Hotta, Y. (1995) Cell 82, 1025–1036
2. Jones, B. W., Fetter, R. D., Tear, G., and Goodman, C. S. (1995) Cell 82, 1013–1023
3. Vincent, S., Vonesch, J.-L., and Giangrande, A. (1996) Development 122, 131–139
4. Schreiber, J., Sock, E., and Wegner, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4739–4744
5. Akiyama, Y., Hosoya, T., Poole, A. M., and Hotta, Y. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14912–14916
6. Altschuller, Y., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Frohman, M. A. (1996) FEBS Lett. 383, 201–204
7. Kammerer, M., Pirola, B., Giglio, S., and Giangrande, A. (1999) Cytogenet. Cell Genet. 84, 43–47
8. Kanemura, Y., Hiraga, S., Arita, N., Ohnishi, T., Izumoto, S., Mori, K., Matsumura, H., Yamashita, M., Fushiki, S., and Yoshimine, T. (1999) FEBS Lett. 442, 151–156
9. Kim, J., Jones, B. W., Zock, C., Chen, Z., Wang, H., Goodman, C. S., and Anderson, D. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 93, 12364–12369
10. Schreiber, J., Enderich, J., and Wegner, M. (1998) Nucleic Acids Res. 26, 2337–2343
11. Basyuk, E., Cross, J. C., Corbin, J., Nakayama, H., Hunter, P., Naing, M., and Lazzerini, R. A. (1999) Dev. Dyn. 214, 303–311
12. Hunter, P. J., Swanson, B. J., Haendel, M. A., Lyons, G. K., and Cross, J. C. (1999) Development 126, 1247–1258
13. Reifegerste, R., Schreiber, J., Gullan, S., Ludemann, A., and Wegner, M. (1999) Mech. Dev. 82, 141–155
14. Kuhlbrodt, K., Schmidt, C., Sock, E., Pingault, V., Bondurand, N., Gossens, M., and Wegner, M. (1998) J. Biol. Chem. 273, 23033–23038
15. Renner, K., Leger, H., and Wegner, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6433–6437
16. Sock, E., Enderich, J., Rosenfeld, M. G., and Wegner, M. (1996) J. Biol. Chem. 271, 17512–17518
17. Wegner, M., Drolet, D. W., and Rosenfeld, M. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4743–4747
18. Akiyama-Oda, Y., Hosoya, T., and Hotta, Y. (1998) Dev. Genes Evol. 208, 578–585
19. Bernardoni, R., Miller, A. A., and Giangrande, A. (1998) Development 125, 3189–3200
20. Miller, A. A., Bernardoni, R., and Giangrande, A. (1998) EMBO J. 21, 6316–6326
21. Rogers, S., Wals, R., and Rechsteiner, M. (1986) Science 234, 364–368
22. Rechsteiner, M., and Rogers, S. W. (1996) Trends Biochem. Sci. 21, 267–271
23. Rabbits, P. H., Watson, J. V., Lamond, A., Forster, A., Stinson, M. A., Evans, G., Fischer, W., Atherton, E., Sheppard, R., and Rabbits, T. H. (1985) EMBO J. 4, 2009–2015
24. Distelhorst, C. W., and Howard, K. J. (1989) J. Biol. Chem. 264, 13080–13085
25. Fuchs, S. Y., and Hanani, Z. (1999) Mol. Cell. Biol. 19, 3289–3298
26. Hanteboer, G., Kerkhoven, R. M., Stibrants, A., Bernard, R., and Beijersbergen, R. L. (1996) Genes Dev. 10, 2960–2970
Protein Stability and Domain Topology Determine the Transcriptional Activity of the Mammalian Glial Cells Missing Homolog, GCMb
Elisabeth E. Tuerk, Jörg Schreiber and Michael Wegner

J. Biol. Chem. 2000, 275:4774-4782.
doi: 10.1074/jbc.275.7.4774

Access the most updated version of this article at http://www.jbc.org/content/275/7/4774

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 26 references, 14 of which can be accessed free at http://www.jbc.org/content/275/7/4774.full.html#ref-list-1