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DNA-dependent conversion of Oct-1 and Oct-2 into transcriptional repressors by Groucho/TLE

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ABSTRACT

POU domain proteins contain a bipartite DNA-binding element that can confer allosteric control of coactivator recruitment. Dimerization of Oct-1 and Oct-2 on palindromic response elements results in the conformational dependent inclusion or exclusion of the transcriptional coactivator OBF-1. In this paper, we demonstrate that Oct-1 and Oct-2 can function as transcriptional repressors by recruiting and physically interacting with members of the Grg/TLE family of corepressors. In accordance with a model of DNA induced cofactor assembly, and analogous to the recruitment of the OBF-1 coactivator, the different Grg/TLE members can discriminate between both Oct-1 and Oct-2, and the monomeric or dimeric nature of the POU/DNA complex.

INTRODUCTION

A central issue in understanding gene transcription is determining how tissue specificity is achieved, when essentially every cell within an organism possess the same genetic information. Certain transcription factors can adopt different conformations on DNA depending on the actual sequence they are binding to. The POU proteins share a conserved bipartite DNA-binding domain consisting of a POU specific (POU_S) and a POU homeodomain (POU_H) tethered by a flexible linker region that varies in length between different members (1). Consequently, POU_S and POU_H can in certain situations behave as separate DNA-binding modules (2). This flexibility allows for the POU domain to bind to DNA in a variety of forms, as recently described for the Oct and Pit transcription factors (3,4).

This divergent sequence recognition has consequences for the recruitment of the Oct-1/Oct-2 coactivator OBF-1 (BOB-1, OCA-B). Oct-1 and Oct-2 were originally discovered by their ability to bind to the octamer motif (5). A second, palindromic Oct binding sequence (PORE: ATTTGAAATGCAAAT) was identified in the enhancer region of the osteopontin gene, which supported Oct dimer formation and again allowed for binding of the OBF-1 coactivator (6). The PORE sequence can support both monomeric and dimeric binding of Oct proteins, seemingly in approximately equal amounts. Subsequently, a second palindromic motif (MORE: ATGCATATGCAT), which excluded OBF-1 binding, was identified in immunoglobulin V region promoters (4).

In this study, we show that the POU domain of Oct-1 and Oct-2 discriminates between different members of the Grg/TLE family of transcriptional repressors. In accordance with the theory that the DNA motif can determine cofactor recruitment (7), we show that the Oct-1 specific repression through Grg/TLE2 and Oct-2 repression through Grg/TLE4 is augmented on dimeric binding motifs.

MATERIALS AND METHODS

Cell lines, plasmid constructs and transient transfections

The cytomegalovirus (CMV)-based eukaryotic expression vectors were obtained from the following investigators. Grg/TLE 1–3 and pB-catenin-DP were obtained from H. Clevers, Grg/TLE 4 was obtained from M. Busslinger, Oct-2 and octamer reporters were obtained from T. Wirth. The C-terminal mutant of Oct-2 was from W. Herr, OBF-1 and the Oct-2 N-terminal deletion, amino acids 189–479, were from P. Matthias. Oct-1 was from W. Schaffner and *Drosophila* Groucho was from Y. Engström. The PORE and MORE reporter constructs were from H. Schöler. Transient transfections were performed on 293A cells in 6 cm plates using the FuGene6 transfection reagent (Roche). The total amount of DNA was kept constant by the addition of an empty CMV containing vector. After 40–48 h, Luciferase

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and β-galactosidase measurements were performed using the TROPIX dual reporter assay kit according to the manufacturer's instructions (Applied Biosystems).

Electrophoretic mobility shift assay

A double-stranded oligonucleotide encompassing a classical octamer from the immunoglobulin heavy chain enhancer 5′-GATCCATCCATTTGCATGTGCCTCGA-3′ was used to detect Oct binding. An Ets protein-binding sequence was used as unspecific competitor: 5′-CTAGCGAGAAATAAAAGGAAGTGAAACCAAGTGCTA-3′. The binding assay was performed in a binding buffer (25 mM HEPES, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$ 0.2 mM EDTA, 1 μg BSA and 4% Ficoll), radiolabelled probe, 0.25 μg poly(dI–dC) (Amersham Biosciences, 27-7880) and ∼5 μg nuclear extract. An aliquot of 1 μg of α-Oct-1 (sc-8024) or α-XBP-1 (sc-7160) antibodies (Santa Cruz) were used for supershifts.

Co-immunoprecipitation

Nuclear extracts were prepared from 293A cells and precleared with Protein A Sepharose CL-4B (40 μl, 50%, Amersham Pharmacia) in IP-buffer (total volume 500 μl/sample, IP-buffer; 20 mM HEPES pH 7.4, 0.1 M NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 2% glycerol, 1% NP-40 and 1 mM phenylmethylsulfonyl fluoride) at 4°C. For immunoprecipitations, supernatants were transferred to new tubes and beads (40 μl, 50%) substituted with antibody, (α-TLE, sc-13373 Santa Cruz), or beads only, were added. The samples were rotated at 4°C overnight. The precipitates were washed and analysed on a 10% SDS–PAGE. Western blots were probed with α-Oct-2 antibody (sc-233, Santa Cruz) Antigen was visualized using the ECL™ detection system (Amersham Pharmacia).

Glutathione *S*-transferase interaction experiments

The fusion protein of glutathione *S*-transferase (GST) with the Oct-2 POU domain was a kind gift from E. Turner and has been described previously (8). Approximately 3 μg of bacterially produced protein was incubated with 3–4 μl of S^{35} *in vitro* labelled proteins in 300 μl of binding buffer (20 mM Tris–HCl, pH 7.8, 0.1% Triton X-100, 0.1 mg/ml BSA, 10% glycerol, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 50 μg/ml ethidium bromide and Complete™ protease inhibitors) for 20 min at room temperature. Precipitates were then washed six times in buffer B (binding buffer minus BSA, ethidium bromide and protease inhibitors). Samples were resolved by running on 10% SDS–PAGE and analysed by a PhosphoImager (Fuji BAS 1800).

RESULTS

Conserved repression of Oct-1 and Oct-2 on classical monomeric octamer sequences

The lymphoid expressed proteins Oct-1 and Oct-2 have been described as repressors of transcription (9,10). However, the underlying mechanism(s) for this repression has not been determined (11). A possible clue comes from the observation that a number of other lymphoid transcription factors interact with members of the Groucho/TLE family, e.g. Pax5 (12) and Pu.1 (13).

To investigate whether Groucho/TLE proteins could function as an interaction partner for Oct proteins, we performed transient transfections using the four full-length Grg/TLE1-4 corepressors on a minimal luciferase construct located downstream of multiple copies of the standard octamer sequence. Activation of the reporters was achieved by the addition of Oct-1 (Figure 1A) or Oct-2 (Figure 1B) and their coactivator OBF-1. As shown in Figure 1A, Grg/TLE1 and 3 could functionally repress Oct-1 transcription to a much greater degree than Grg/TLE2 and Grg/TLE4. This experiment was then repeated using Oct-2 and the same pattern of repression was seen, with the exception that the limited repression seen with Grg/TLE2 on Oct-1 was not observed (Figure 1B).

To confirm that the Grg/TLE expression plasmids used were functional, a control experiment using an unrelated Grg/TLE-responsive promoter was performed. A construct containing TCF-responsive elements, which are known to be repressed by all Grg/TLE proteins (14), was analysed. As expected, the activation induced by TCF-1 together with β-catenin could be efficiently repressed with all four of the Grg/TLEs (Figure 1C). Finally, expression levels of the four myc-tagged Grg/TLE proteins were determined by analysing the nuclear extracts used in Figure 1D. As demonstrated in Figure 1D, all four proteins are expressed, with Grg/TLE3 being the most pronounced.

In conclusion, transcription activation by Oct-1 or Oct-2 in conjunction with OBF-1 can be prevented by Grg/TLE1 and 3. In contrast, Grg/TLE2 and 4 had a limited affect on both Oct proteins, despite of the fact that both of these repressors are functionally expressed.

Divergent responses of Oct-1 and Oct-2 to Grg/TLE on dimeric response elements

Having shown that Grg/TLE1 and 3 are functionally capable of repressing Oct-1 and Oct-2 driven transcription on an octamer reporter, we next determined whether the PORE element, which allows for dimeric Oct binding, would respond differently to Grg/TLE members. Therefore, the experiment was repeated using the PORE-D-based reporter. The PORE-D sequence differs from the PORE sequence discovered in the osteopontin enhancer in that it only supports dimeric, and not monomeric Oct binding. The wild-type PORE sequence retains some competency in supporting monomeric binding as well (6). In contrast to the octamer reporter, only Grg/TLE2 suppressed transcriptional activation (Figure 2A). Importantly, in spite of the greater expression levels of Grg/TLE3 (Figure 1D), compared with the other three members, this was still not sufficient to facilitate repression on Oct-1 on the PORE sequence. In sharp contrast, when the experiment was repeated using Oct-2 (Figure 2B), a different pattern of responses was noted. Although Grg/TLE1 and 3 still repressed, in addition so could Grg/TLE4. The response profile of Oct-2 transcriptional activation on the PORE-D was, therefore, the exact opposite of Oct-1, which was only responsive to Grg/TLE2.

To further confirm that there is a conformational dependency between Oct-2 and Grg/TLE4, a comparison of three different promoters was used: the octamer (monomeric), the PORE (monomeric and dimeric) and the PORE-D (dimeric).

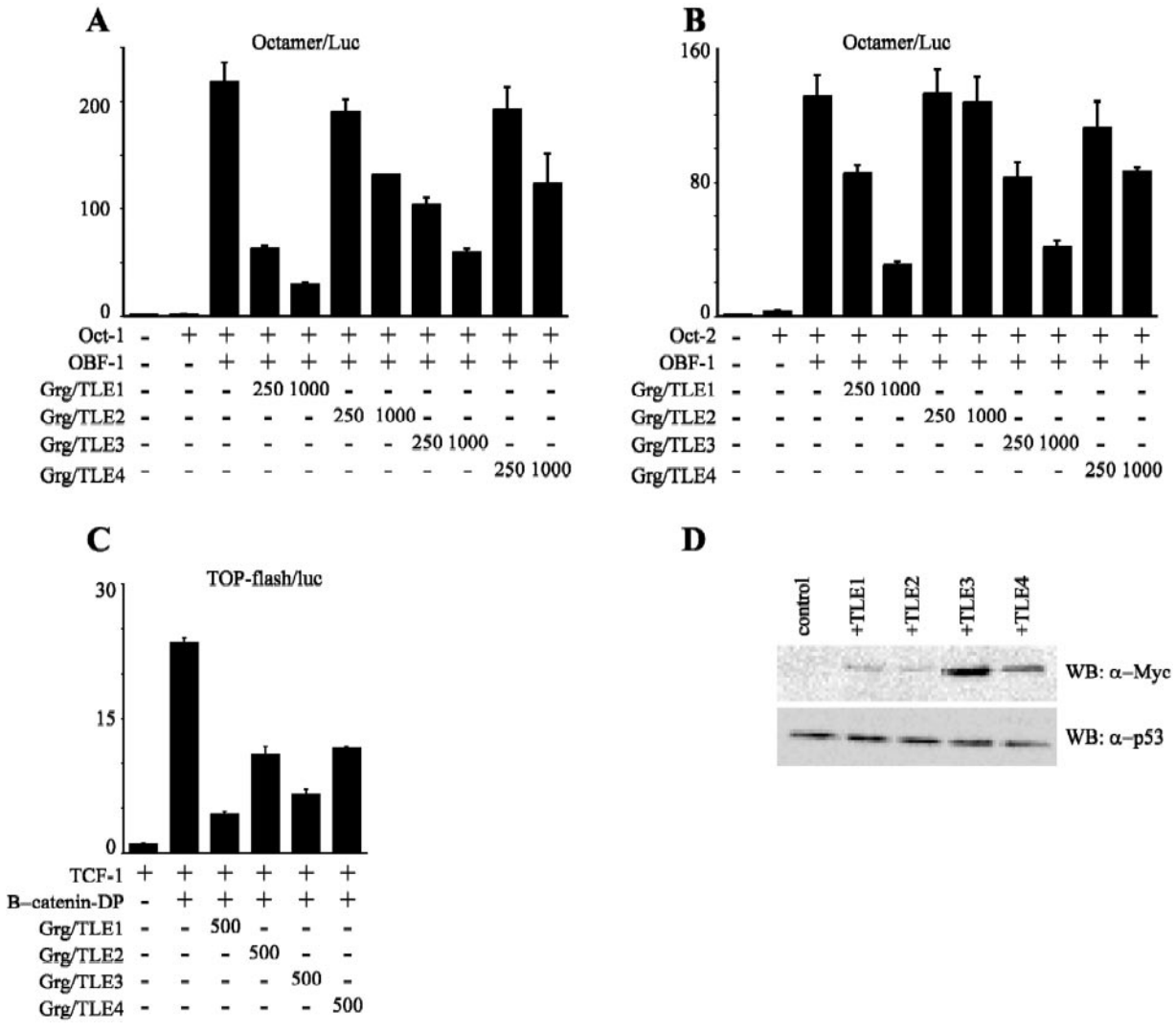

Figure 1. Grg/TLE proteins can repress Oct-1 and Oct-2. Repression of Oct-1 (**A**) and Oct-2 (**B**) on the octamer. 293A cells were transfected with 1 μg reporter containing four copies of the consensus Octamer Sequence 5′-ATGCAAT-3′ linked to tk-promoter luciferase gene. The promoter was activated with 0.5 μg of CMV-driven eukaryotic expression vectors for Oct, 2.0 μg of OBF-1 and the indicated amount of Grg/TLE proteins. As a transfection control, 10 ng of CMV-based β-galactosidase was included. The *y*-axis indicates fold activation relative to the activity of the reporter alone, which was arbitrarily set to 1, as determined as the ratio between luciferase and β-galactosidase measurements. The figure represents triplicate experiments with ±SD indicated on the error bars. All Grg/TLE members can repress TCF-1/β-catenin dependent activation (**C**). In this experiment, 1 μg of reporter was transfected together with 0.5 μg of TCF-1, 0.1 μg of constitutively active β-catenin and the 500 ng of each Grg/TLE. Expression of Grg/TLE (**D**). Nuclear extracts were probed for myc-tagged Grg/TLE (α-Myc). An antibody against p53 was used as a protein loading control.

If Grg/TLE4 expresses a preference for dimeric binding sites, then one would expect an increase in repression from octamer to PORE, and from PORE to PORE-D. Figure 2C shows that this clearly happens. Although there is some down-regulation on the octamer, as indicated in Figure 1B, this is greatly augmented on the PORE. Furthermore, an increase in repression is seen on the PORE-D when compared with the PORE.

In conclusion, Grg/TLE proteins show a complex pattern of repression on Oct-1 and Oct-2, depending on the binding site examined. These patterns are tabulated in Figure 2D. Of particular interest is that seen on the dimeric PORE-D sequence, where Grg/TLE2 can only repress Oct-1 and not Oct-2, whereas Grg/TLE4 seems only to act on Oct-2 and not Oct-1.

Finally, we wished to determine how Oct-1 would respond to Grg/TLE proteins in the context of a naturally occurring regulatory element, as opposed to the artificial MORE and PORE constructs, which contain multiple binding sites for Oct factors. We tested the four Grg/TLE corepressors on a Vh PORE region derived from the immunoglobulin heavy chain promoter locus, containing one binding site for Oct-1 or Oct-2 (4). Surprisingly, this reporter responded differently to the multimerized constructs in that Grg/TLE1 and 3, but not Grg/TLE2, were capable of functional repression (Figure 2E).

The POU domain of Oct-2 interacts with the SP domain of Grg/TLE4

To further investigate the interaction between Oct-2 and Grg/TLE, we specifically assessed the conformationally recruited Grg/TLE4. The ability to distinguish between different *cis* motifs suggested to us that the recruitment of

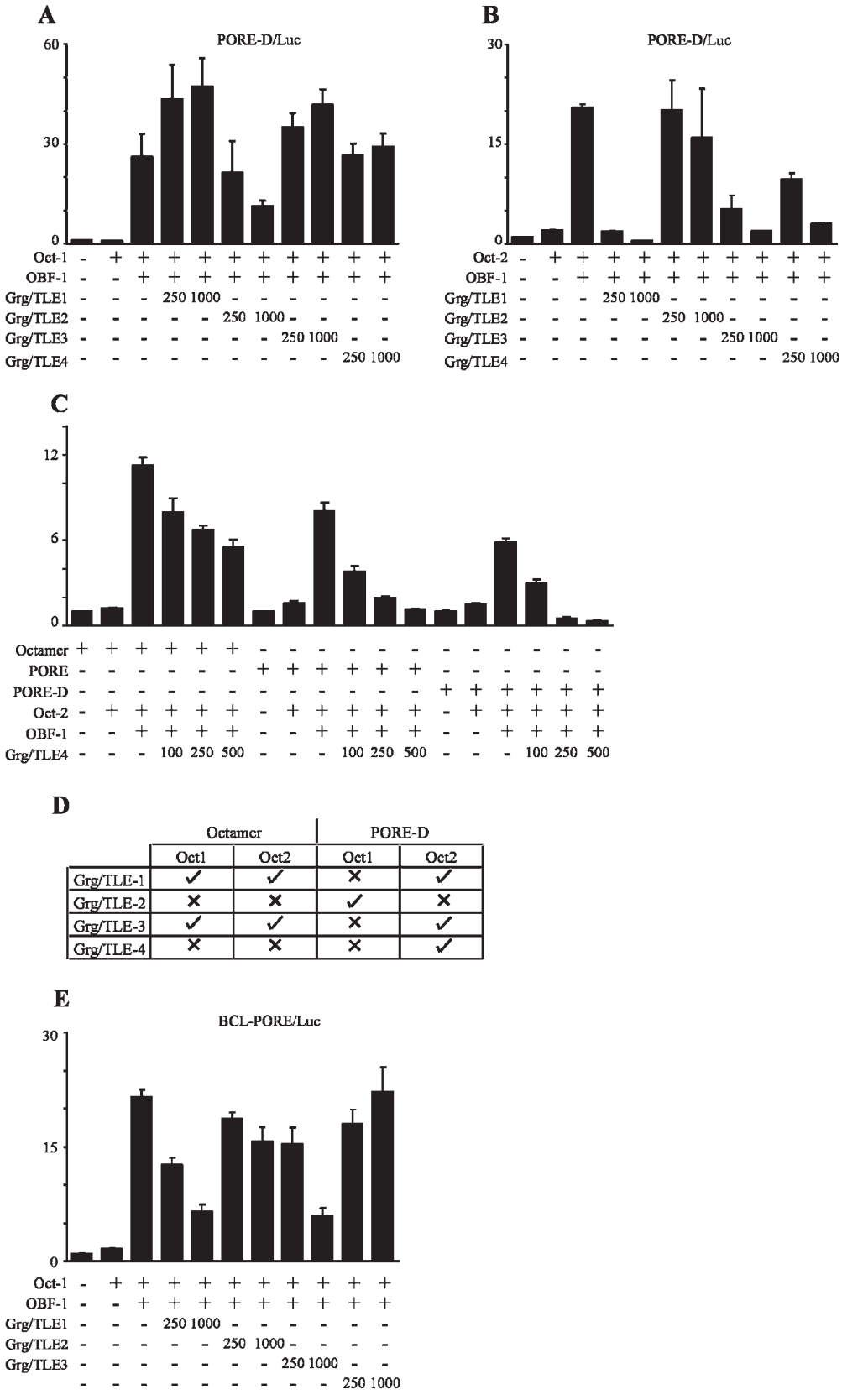

Figure 2. Differential repression patterns on dimeric sequences. Transient transfections on PORE-D sequence with Oct-1 (**A**) and Oct-2 (**B**). Increasing repression from monomeric to dimeric reporters (**C**). In this experiment, fold activation is determined with respect to the level of the reporter alone, for each of the three separate constructs. (**D**) A summary of the repression pattern for the experiments from Figures 1 and 2. A tick indicates repression by the indicated Grg/TLE, whereas a cross indicates relatively normal transcriptional activation (**E**) A Vh BCL-PORE Promoter can also be targeted by Grg/TLE.

Grg/TLE could occur through the POU domain. However, the N-terminal region of Oct has previously been shown as a repressor of a number of different promoters (15,16). To exclude the N-terminal repression region as being the Grg/TLE interacting domain, we assessed different deletion mutants of Oct-2. Removal of the N-terminal (Oct-2ΔN) still permitted repression, as did deletion of the C-terminal (Oct-2ΔC), although to a lesser extent, while Oct-1 was still refractory the effects of Grg/TLE4 (Figure 3A). Therefore, the previously reported repression domain in the N-terminus of Oct-2 is unlikely to recruit corepressors of the Grg/TLE family.

We next wished to determine whether Oct-2 and Grg/TLE complex formation occurs *in vivo*. Grg/TLE proteins were immunoprecipitated from 293A cells, a non-lymphoid cell line that does not express Oct-2 but does produce Grg/TLE proteins (14). Immunoprecipitation with a pan-Grg/TLE antibody, but not protein A alone, resulted in Oct-2 only being co-precipitated when Oct-2 had been transfected into the cell line (Figure 3B), indicating that over expressed Oct-2 interacts with endogenous Grg/TLE proteins. To further confirm that Oct-2ΔN could interact with Grg/TLE members, immunoprecipitation was performed again and Oct-2ΔN could indeed be precipitated with endogenously expressed Grg/TLE proteins.

The functional and immunoprecipitation data indicated an interaction between Oct-2 and Grg/TLE4; we therefore wished to determine the binding domain(s) responsible. First, Grg/TLE4 lacking various C-terminal domains were assessed for the ability to repress Oct-2/OBF-1 potentiated transcription on the PORE-D driven promoter (Figure 3C). The removal of the most C-terminal region, the WD40 repeats, had no effect on the repression process. However, subsequent removal of the next C-terminal region, the SP domain, alleviated the Grg/TLE4-mediated transcriptional repression. Western analysis also confirmed that these deleted mutants were stably expressed (Figure 3D).

Figure 3C indicates that the SP domain appears to be crucial for the formation of a functional repression complex with Oct-2. To determine whether the interaction between Oct-2 and Grg/TLE4 is direct, GST pull-down reactions were performed. GST, or GST fused to the POU domain of Oct-2 was used as bait for *in vitro* radiolabelled Grg/TLE4 and a mutant lacking the WD40 and SP domain. As shown in Figure 3E, GST-POU, but not GST alone could interact with full-length Grg/TLE4. In contrast, only very low levels of Grg/TLE4-ΔSP could be detected.

Grg/TLE prevents Oct-driven transcription and the interaction is evolutionarily conserved

The removal of the inhibitory N-terminal region generates an Oct-2 mutant, which is both competent for Grg/TLE-mediated repression and has greatly elevated transcriptional activation properties, as previously demonstrated (15). This allowed us to determine whether repression could occur in the absence of OBF-1, on both the PORE-D and the OBF-1 excluding MORE motif. Using transient transfections, we demonstrated that Grg/TLE4 could efficiently extinguish OBF-1 independent, Oct-2 driven transcription on both a PORE-D and a MORE driven reporter (Figure 4A).

Both *Drosophila* Groucho and Grg/TLE in certain situations mediate repression through interaction with Histone deacetylases (HDAC) (14,17). Transfections performed in the presence of the HDAC inhibitor Trichostatin A (Figure 4B) failed to relieve repression. Therefore, at least in the case of Oct-2 and Grg/TLE4, an HDAC-independent mechanism is in operation.

As the underlying repression mechanism seems to a level distinct from HDAC recruitment, it is possible that Grg/TLE prevents DNA binding of Oct proteins. EMSA analysis using endogenously expressed Oct-1 and transfected OBF-1 indicated that Oct-1 is fully competent in both binding DNA and forming a stable DNA/Oct-1/OBF-1 ternary complex in the presence of Grg/TLE1 (Figure 4C). This strongly suggests that Grg/TLE-mediated repression is not simply sequestering of Oct from its DNA target. Therefore, the Grg/TLE-mediated transcriptional repression prevents a fully active Oct-1/OBF-1 complex from successfully transcribing a reporter gene.

Oct-2 is part of large conserved family of POU domain proteins (18), and an interaction with *Drosophila* Groucho may be indicative of a greater evolutionary relationship between POU domains and Grg/TLE/Groucho corepressors. GST pull-down experiments indicate that *Drosophila* Groucho can directly interact with the POU domain of Oct-2 but not with GST alone (Figure 4D). In concordance with this data, Groucho was able to repress Oct-2/OBF-1-mediated transcriptional activation (Figure 4E). Given that the interaction between Oct-2 and Grg/TLE/Groucho is evolutionary conserved, it will be interesting to determine whether other POU domain containing factors can also interact with the Grg/TLE family of corepressors.

DISCUSSION

In this paper, we demonstrate a complex interaction between a transcriptional corepressor (Grg/TLE) with a transcriptional activator (Oct), on divergent DNA-binding sites. Therefore, Grg/TLE proteins are the first described transcriptional corepressors for both Oct-1 and Oct-2. A tabulation of these interactions is shown in Figure 2D. Of particular interest is the conformational dependency exhibited on the octamer motif, for Oct-1 and Grg/TLE2, and on the PORE-D motif, for Oct-2 and Grg/TLE4. However, it would appear that these interactions are dependent on the context and copy number of the binding site. As shown in Figure 2E, a single binding site for Oct, which is a common occurrence within the promoters of immunoglobulin genes, displays an alternate pattern of repression in response to Grg/TLE. Regardless of these differences in promoter responses, other regulatory regions, which are known to be repressed by Oct transcription factors, may do so through interaction with the Grg/TLE family of corepressors.

This is not the first example of Oct-1 and Oct-2, which have highly conserved DNA-binding domains, showing such discrimination in the recruitment of cofactors. For example, in spite of POU domain sequence similarity, only Oct-1 can support binding of the viral coactivator VP16. This interaction is due to the presence of a single amino acid that is absent in Oct-2 (19).

A conformational mechanism for the recruitment of the Grg/TLE2 corepressor to the POU domain of Oct-1,

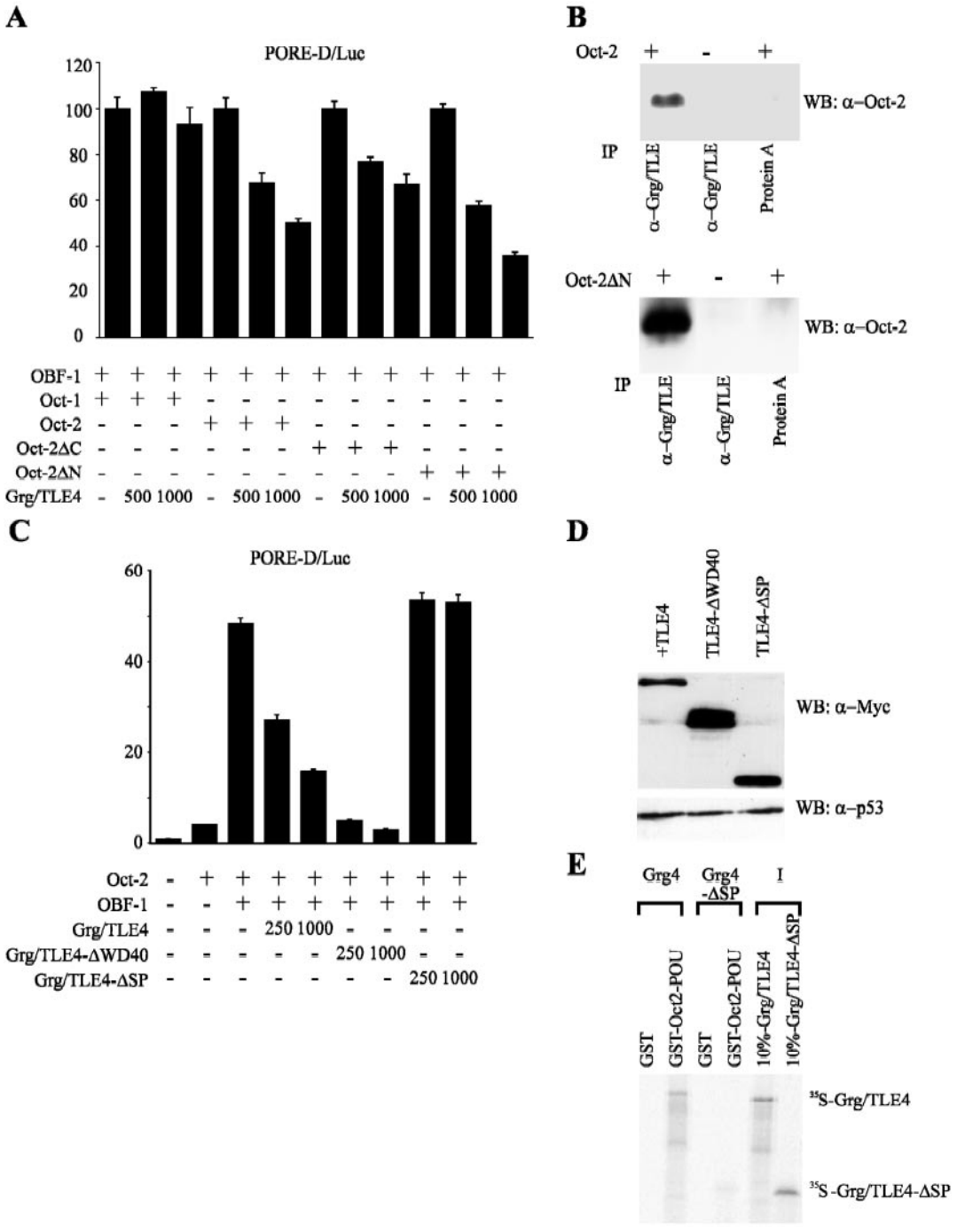

Figure 3. The POU domain of Oct-2 interacts with the SP domain of Grg/TLE4. The N-terminal deletion of Oct-2 (Oct-2ΔN) lacking the first 187 amino acids of Oct-2 is efficiently repressed by Grg4 as is the C-terminal mutant Oct-2 (Oct-2ΔC) on the PORE-D reporter (**A**). In this experiment, activation with Oct/OBF-1 alone is set to 100% for each of the four constructs and the *y*-axis represents percent activation. Co-immunoprecipitation of Oct-2 with endogenous Grg proteins (**B**). Top: 2 μg of Oct-2 plasmid was transiently transfected into 293A cells. Nuclear extracts were incubated with an anti-TLE antibody recognizing Grg/TLE1-4 and western blots probed with an Oct-2 specific antibody. Bottom: the N-terminal mutant Oct-2, Oct-2ΔN, interacts with endogenous Grg/TLE. Cotransfection of 293A cells with various mutants of Grg4 (**C**). The SP domain is required for repression. The Grg/TLE4-ΔWD40 construct is lacking the WD40 domain. The Grg/TLE4-ΔSP mutant is devoid of both the WD40 domain and the SP domain. (**D**) Western analysis of myc-tagged Grg4 and mutants to show stable expression. (**E**) GST pull-down indicates that the POU domain interacts with the SP domain of Grg4, but not with the SP mutant (Grg4-ΔSP). I indicates 10% input lanes.

and Grg/TLE4 to the POU domain of Oct-2, extends our understanding of how specificity may be achieved in the regulation of transcriptional activity. Strikingly, the evolutionary conserved POU domain has evolved so that different members are able to discriminate between individual Grg/TLE corepressors, as demonstrated here by Oct-1 and Oct-2. Furthermore, the interaction of this POU domain with specific DNA sequences can, in the case of Grg/TLE2 and Grg/TLE4,

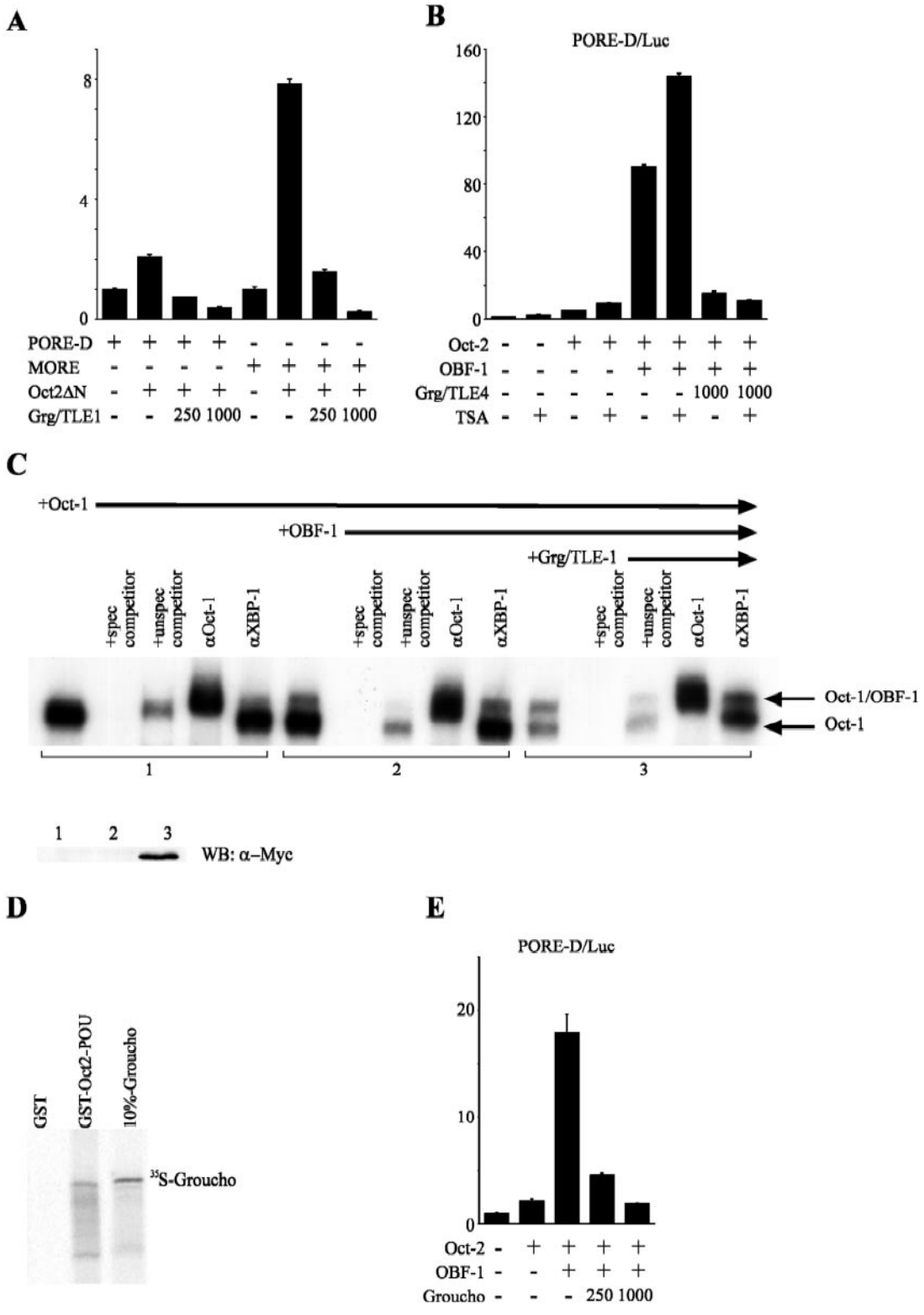

Figure 4. The evolutionary conserved interaction prevents Oct transcription. (**A**) Oct-2ΔN can be repressed by Grg/TLE1 on both the PORE-D and MORE sequences. (**B**) Transfections preformed in the presence of TSA (50 ng/ml) fails to relieve repression. (**C**) EMSA analysis of the Oct/OBF-1/DNA ternary complex. Endogenous Oct-1nuclear extracts (1) together with OBF-1 (2) and OBF-1 with Grg/TLE1 (3) were assessed for their ability to bind to octamer sequence labelled probe. The complex was shifted by αOct-1 but not by an unspecific antibody (XBP-1). Note the formation of the Oct/OBF-1 complex in both nuclear extracts 2 and 3. Below is a western blot of extracts 1–3 showing the presence of transfected Grg/TLE only in extract 3. GST pull-down experiments using the POU domain of Oct-2 indicate a direct interaction with *Drosophila* Groucho (**D**). *Drosophila* Groucho can repress Oct-2 mediated activation on the PORE-D promoter (**E**).

determine whether the corepressor is recruited to a regulatory unit. Finally, the data presented here may be applicable to a more generalized methods for transcription factor assembly and subsequent transactions (20,7).

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