Transcriptional activation by Oct-3: evidence for a specific role of the POU-specific domain in mediating functional interaction with Oct-1

Maria Alessandra Vigano* and Louis M. Staudt

DIBIT–Istituto Scientifico H.S.Raffaele, Milano, Italy and 1Metabolism Branch, NCI, NIH, Bethesda, MD, USA

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ABSTRACT

Oct-3, a member of the POU family of transcription factors, is expressed in pluripotent cells of early mammalian embryos and in undifferentiated embryonal carcinoma cell lines. Using a variety of Oct-3 mutants, we have identified two different domains of Oct-3 which activate transcription in transfected mammalian cells. One of these domains, located in the C-terminal part of the protein, plays a major role in transcriptional activation when Oct-3 is bound to its cognate site, the octamer motif. An Oct-3 mutant containing a single amino acid substitution in the POU homeodomain is unable to bind the octamer target in vitro, yet is still able to activate transcription in an octamer-dependent manner. We provide evidence that transactivation by this mutant involves protein–protein interactions with the ubiquitous octamer binding factor Oct-1. This interaction requires the POU-specific domain of Oct-3 and allows recruitment of Oct-3 to the target promoter even in the absence of Oct-3 DNA binding.

INTRODUCTION

The octamer cis-acting transcriptional regulatory motif (ATGC-AAAAT) is found in enhancers and promoters of many genes which are expressed either ubiquitously or in a tissue-specific fashion (1–7). The octamer motif regulates gene expression by the binding of transcription factors belonging to the POU family (8–10). These factors are characterized by the presence of the conserved 160 amino acid POU domain, a bipartite DNA binding structure containing a 74–82 amino acid N-terminal POU-specific region (POUS) and a 60 amino acid POU homeodomain (POU HD), connected by a 15–27 amino acid linker region (9–12). The POU HD shares ~33% amino acid identity with the homeodomains of the Antennapedia class of homeoproteins. Furthermore, its tertiary structure, as determined by X-ray crystallography (13), is very similar to the structures of other homeodomains. DNA binding by the POU domain involves interaction of the third ‘recognition’ helix of the POU HD with bases in the major groove. The POUS domain is required for site-specific, high affinity DNA binding and bending (14) and contacts DNA in the 5′-half of the octamer motif using a helix–turn–helix structure similar to that of the λ and 434 repressors (15–17).

In addition to their DNA binding function, both the POUS and the POU HD can participate in protein–protein interactions with both POU proteins and other transcriptional regulators. Homo- and heterodimerization, mediated by the POU domain (20), has been demonstrated for several POU proteins binding to multiple adjacent sites. For example, POU domain interactions promote the cooperative binding of Pit-1 and Oct-1 to the Pit-1-responsive element of the prolactin promoter (23). Protein–protein interactions mediated by the POU domain are important for both activation and repression of transcription. For example, Oct-1 is able to bind to the herpes simplex virus transactivator VP16 through specific residues of the POU HD, leading to activation of the viral immediate early genes (24–29), whereas specific interaction between helix 1 and 2 of the POU HD of the Drosophila proteins I-POU and Cf1a leads to specific inhibition of transactivation of the DOPA decarboxylase gene (30,31).

The expression of most POU proteins is regulated during embryogenesis, as revealed by in situ hybridization studies, which reflects their critical roles in development and cell type determination (11,12,32,33). Expression of one of these genes, Oct-3 (also termed Oct-4) is restricted to the pluripotent cells of the blastocyst during early stages of embryonic development and it is confined to the primordial germ cells after gastrulation (34–37). Oct-3 is also expressed in embryonal stem cells and teratocarcinoma cell lines and its expression is down-regulated after induction of differentiation with retinoic acid. This suggests a role for Oct-3 in maintaining the undifferentiated state of multipotent embryonic cells (34,36–39).

The Oct-3 protein can activate transcription from octamer-containing promoters (34,35,37,39). By fusion of Oct-3 domains with heterologous DNA binding domains, a transactivator domain has been mapped within the proline-rich N-terminal region (39,40). In this report, we show that in the context of octamer-mediated transcriptional activation, the strongest activation domain of Oct-3 actually lies within the C-terminal part of the protein, while the N-terminus has a much weaker activity. Furthermore, we present evidence that protein–protein interactions mediated by the POUS domain with at least another POU protein,
the ubiquitous factor Oct-1, can be functionally relevant for the octamer-specific transcriptional activating function of Oct-3.

**MATERIALS AND METHODS**

**Cell culture and transfection**

F9, HeLa and NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and L-glutamine, in a 5% CO₂ humidified atmosphere at 37°C. For transfection, cells were seeded in 100 mm tissue culture dishes, allowed to reach 1/3–1/2 confluency and transfected using the calcium phosphate precipitation method with 20 μg total DNA (2 μg reporter plasmid, 0.13 μg reference plasmid, 0.5–4.0 μg transactivator expression plasmid and with pUC19 plasmid added to bring the total DNA to 20 μg), according to standard procedures (41). The precipitate was removed 15–20 h after transfection by extensive washing with PBS and PBS containing 2 mM EGTA. Two days after transfection, the cells were harvested for cytoplasmic RNA or protein extraction.

**Plasmid construction**

**Expression vectors and mutants.** The PCG-Oct-3 plasmid was made by inserting the NcoI–BclI fragment of the Oct-3 cDNA into the PCG mammalian expression vector (34,42). Mutations in the Oct-3 coding sequence were generated either by site-directed mutagenesis (43) or by PCR and always verified by Sanger DNA sequencing (Sequenase; US Biochemical). The 5′–3′ construct had a deletion in the N-terminal, proline-rich region from Pro13 to Pro60. The 3′ construct had a deletion in the C-terminal region from Gin287 to Ser320. The POU construct contained the entire POU domain of Oct-3, from Glu127 to Ser272. The ‘frameshift’ construct, which destroyed the Oct-3 open reading frame at 91 bp from the starting ATG, was generated by digestion of PCG-Oct3 with BamHI, blunting with Klenow polymerase and religation. The Gal4–Oct–3′-5′ fusion plasmid contains the 5′ EcoRI–PstI Oct-3 fragment inserted at the Smal site of the Gal4-1–147 expression plasmid (44). The Gal4–E1α expression plasmid has been described previously (45).

**Reporter and reference plasmids.** The reporter plasmid containing wild-type (B20 dpm2) and the reporter plasmid containing mutant (B20 dpm8) octamer motifs have been previously described (42). In these plasmids, six copies of the synthetic SV40 B element, containing a wild-type or mutant octamer motif, were cloned upstream of the human β-globin gene. The internal reference plasmid contains four tandem copies of the A and C SV40 enhancer elements inserted downstream of the α-globin gene (42). The Gal4x1–E1b and Gal4x5–E1b reporter plasmids were described previously (45) and contained one or five Gal4 binding sites, respectively. A RSV–β-gal expression vector was used for normalization of transfection efficiency in some experiments.

**RNA and protein analysis**

Cytoplasmic RNA was extracted from transfected cells following NP-40 lysis (41). Fifteen or 20 μg RNA were hybridized overnight at 45°C to antisense α- and β-globin riboprobes (5 × 10⁶ c.p.m.), digested with an RNase A and T1 mixture and run through a denaturing 5% polyacrylamide gel (41). The correctly initiated α- and β-globin transcripts gave rise to protected bands of 132 and 350 bp, respectively. Every experiment was performed at least in triplicate and the results quantitatively analysed using a PhosphorImager (Molecular Dynamics).

Total cell extracts were prepared from transfected cells as described (41). Electrophoretic mobility shift assays were carried out as described (46), using a gel-purified, end-labelled double-stranded oligonucleotide probe derived from the Ig-κ promoter and 3 μg cell extract.

For Western blot analysis, 30 μg transfected cell extract were electrophoresed through a 10% SDS–polyacrylamide gel, electroblotted onto a nitrocellulose membrane, incubated with an anti-Oct-3 rabbit polyclonal antiserum raised against a bacterially expressed protein and visualized with 125I-labelled protein A (41).

**Protein–protein affinity chromatography**

The POU-specific region of Oct-1, generated by PCR, was cloned in-frame into the Xmal site of the pGex 2T vector (Pharmacia). The glutathione S-transferase fusion protein (GST–Oct–1 POU₃) and GST alone were expressed in Escherichia coli according to established methods (41). Briefly, fresh cultures of bacteria containing either pGex 2T or the fusion construct pGex-Oct1 POUS were induced for 3 h with 0.5 mM IPTG. Cells were harvested by centrifugation, resuspended in MTPBS (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, pH 7.3) containing 1% Triton X-100 and 1 mM PMSF and sonicated for 20 s (low energy, Branson Sonifier). The supernatants were incubated for 10 min at 4°C with glutathione–Sepharose 4B resin (Pharmacia) on a rotating wheel. After three washes with MTPBS, the resin, bearing approximately equal amounts of either GST or GST–Oct1 POUS (>90% pure as determined by Coomassie staining on an SDS–polyacrylamide gel), was incubated for 1 h at 4°C on a rotating wheel with in vitro translated, 35S-labelled Oct-3 protein in 200 μl HND binding buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 0.1% NP-40, 5 mM DTT, 10 mg/ml BSA) in the presence of 200 μg/ml ethidium bromide. The Oct-3 protein was generated by in vitro transcription, using T7 RNA polymerase, and subsequent in vitro translation, using rabbit reticulocyte lysates, according to the manufacturer’s procedure (Promega). After four washes of 5 min each at 4°C with MTPBS containing 0.1% NP-40, the resin was resuspended in SDS–PAGE sample buffer and electrophoresed through a 10% SDS–PAGE gel. The gel was treated for fluorography with Entensify*A and Entensify*B solutions (DuPont), dried and exposed to Kodak X-AR films at −70°C with an intensifying screen.

**RESULTS**

The major transcriptional activation domain of Oct-3 resides in the C-terminal region

To characterize the domains of the Oct-3 protein required for octamer-dependent transcriptional activation, expression constructs for full-length Oct-3 and various deletion mutants of Oct-3 were co-transfected into HeLa cells with a reporter plasmid containing six copies of the octamer motif upstream of the β-globin promoter/gene. The transcriptional activity of the various Oct-3 mutants was analysed using an RNase protection assay of mRNA from transfected cells. As shown in Figure 1A, removal of the N-terminal, proline-rich region (5P–) had no significant effect on the transactivation activity of the protein.
DNA binding by Oct-3 is not essential for its ability to stimulate transcription

To test whether DNA binding by Oct-3 was required for its transactivation of an octamer-containing target, we inactivated Oct-3 DNA binding by changing a single amino acid (Val235→Pro) in the recognition helix of the POU HD (V-P construct). A similar mutation was previously shown to abolish the binding of Pit-1 to its target sequence (47). As expected, the mutant failed to bind the octamer DNA probe (Fig. 2A, lane 8), although the protein was efficiently produced in transfected cells (Fig. 2B, lane 6). Surprisingly, co-transfection of the V-P mutant with the octamer reporter plasmid resulted in a significant activation of transcription (Fig. 2C, lane 7), ~50% of that observed with the wild-type Oct-3 protein (Fig. 2C, compare lanes 4 and 7). The activity of the V-P mutant was nevertheless dependent on the presence of an intact octamer motif in the reporter construct, since both the wild-type Oct-3 and the V-P mutant failed to activate expression of a construct containing six copies of a mutated octamer element (Fig. 2C, lanes 5 and 8) or a promoter lacking an octamer motif (Fig. 2C, lanes 6 and 9). None of these reporter plasmids were transactivated by the frameshift construct (Fig. 2C, lanes 1–3).

The above findings suggested the existence of additional factors in the transfected cells which were able to direct octamer-dependent transactivation activity from an Oct-3 mutant unable to bind the octamer sequence.
Oct-3 can interact with the Oct-1 POU\(_5\) domains

The above transactivation assays were performed in HeLa cells, which contain Oct-1 as the sole octamer binding protein. Oct-1, by itself, is unable to transactivate the octamer reporter construct used in these assays (Fig. 4C, lane 1; 42). However, we considered the possibility that Oct-1 might interact with the transfected Oct-3 and target it to the reporter construct even in the absence of Oct-3 DNA binding activity. To provide in vitro evidence for protein–protein interactions between Oct-1 and Oct-3, the POU\(_5\) domain of Oct-1 was expressed as a fusion protein with GST in bacteria. The fusion protein was adsorbed to a glutathione–Sepharose resin and then incubated with in vitro translated, radiolabelled Oct-3 protein. The reactions were performed in the presence of ethidium bromide, to exclude potential artifacts related to non-specific binding of the proteins to nucleic acids in the binding reactions (9,48). The proteins retained by the GST–Oct-1 POU\(_5\) resin were analyzed by SDS–PAGE. Figure 3 shows that ~30% of the labelled Oct-3 specifically associated with the fusion protein, while no binding was observed to the GST control protein (Fig. 3, lanes 2 and 3).

Oct-3 functionally interacts with Oct-1 in octamer-dependent transcriptional activation

To provide in vivo functional evidence of an interaction between Oct-3 and Oct-1, we tested whether the activity of the Oct-3 V-P mutant protein could be blocked by co-expression of either the Oct-1 POU\(_5\) domain or the Oct-3 POU\(_5\) domain. As shown in Figure 4A, expression of the isolated Oct-3 POU\(_5\) domain in transfected HeLa cells completely abolished the ability of the Oct-3 V-P protein to activate transcription from the octamer reporter construct. A similar blocking effect was observed when the Oct-1 POU\(_5\) domain was expressed, but not when the Oct-3 frameshift mutant was tested (Fig. 4B, lanes 3 and 4). The ability of an isolated Oct-1 and Oct-3 POU\(_5\) domain to interfere with the...
transcriptional activity of the Oct-3 V-P protein was consistent with the hypothesis that the Oct-3 V-P protein and the endogenous Oct-1 protein interacted through their respective POU domains.

These experiments suggested that the Oct-3 POU domain was necessary for targeting of the Oct-3 V-P protein to the reporter construct. To provide further support for this notion, we constructed a variant form of Oct-3 V-P in which the POU domain was deleted, but the POUHD domain was retained. This protein (Oct-3 V-P POU–) was unable to activate transcription through the octamer motif (Fig. 4C, lane 3), again pointing to a critical role for the Oct-3 POU domain in this phenomenon.

We were next interested in whether the Oct-3 POU domain was sufficient for this targeting. To test this notion, we constructed a plasmid which expressed a fusion protein between the Oct-3 POU domain and the acidic transcriptional transactivator domain of the herpes simplex virus protein VP16 (Oct-3 POU A.A.). Transfection of HeLa cells with this construct together with the octamer reporter plasmid revealed that the Oct-3 POU A.A. chimera protein was able to activate transcription (Fig. 5, lane 1) at a level comparable with, if not higher than the transactivation observed for wild-type Oct-3 (Fig. 5, compare lanes 1 and 3). Furthermore, the transactivation by Oct-3 POU A.A. was octamer specific, since no activity was observed when using a reporter plasmid that contained mutant octamer motifs (Fig. 5, lane 2). Transfection of the Oct-3 POU domain alone did not transactivate the reporter gene (data not shown). Since the POUHD is required for high affinity binding of the POU domain to the octamer motif (9–11,15), the Oct-3 POU A.A. protein would not be expected to bind alone to the reporter plasmid. Rather, the most likely interpretation of this experiment is that the Oct-3 POU domain of the Oct-3 POU A.A. fusion protein interacted with endogenous Oct-1 in the transfected cells, which was able to tether the fusion protein to the octamer reporter plasmid.

DISCUSSION

Oct-3 has been shown to be a potent transcription factor when tested on octamer-containing target sequences (34–37,49). Previous analyses of Oct-3 functional domains outside the POU region suggested that the N-terminal region of the protein, which is rich in proline residues, is responsible for its transcriptional activating function (39,40). This conclusion was drawn from experiments in which the Oct-3 proline-rich region was fused to a heterologous DNA binding domain derived from the c-Jun protein and the resulting chimera was able to activate transcription of a reporter construct containing c-Jun binding sites. We show here that the same N-terminal region of Oct-3 fused to the Gal4 DNA binding domain is able to activate transcription from a reporter gene driven by multimerized Gal4 binding sites, albeit at a low level when compared with the activator domain of the E1a protein. These results confirm the potential activating function of the proline-rich domain of Oct-3 when assayed in the context of a heterologous DNA binding domain. Interestingly, however, we obtained a substantially different result when we analysed the transcriptional activity of Oct-3 when bound to an octamer motif through its own POU DNA binding domain. In this assay, the
that previous studies of the POU S domain have shown that it has a very low affinity for DNA (9,15,20). The transcriptional transactivation of the octamer-containing reporter construct used in transfected HeLa cells. Oct-1, by itself, is unable to activate was conferred by interaction of Oct-3 V-P with Oct-1 in the hypothed that the octamer-dependent activity of Oct-3 V-P activity of Oct-3 V-P was dependent on the presence of an intact octamer binding sequence in the target promoter. We therefore propose that Oct-1, while bound to an octamer motif in a complex to strongly activate transcription using the Oct-3 POU HD, thus accounting for its inability to bind DNA (358). A mutant Oct-3 protein which was unable to bind DNA was nevertheless able to activate transcription from an octamer-dependent reporter gene. This Oct-3 mutant, Oct-3 V-P, has a unanticipated second activation domain which is responsible for most of the transcription activity of the wild-type Oct-3 protein bound to an octamer DNA motif.

A surprising result of our mutational analysis of Oct-3 was that a mutant Oct-3 protein which was unable to bind DNA was nevertheless able to activate transcription from an octamer-dependent reporter gene. This Oct-3 mutant, Oct-3 V-P, has a valine to proline substitution within the recognition helix of the POU HD, thus accounting for its inability to bind DNA in vitro. Although it has been previously shown that the Oct-1 POU domain alone can still bind to DNA, this interaction is of very low affinity and was only detectable using high concentrations of purified POU domain, under assay conditions that stabilized weak interactions (9,15,20,50). Since Oct-3 V-P transactivated the octamer reporter gene ~50% as well as wild-type Oct-3, we consider it unlikely that any residual DNA binding by the Oct-3 POU domain of Oct-3 V-P was responsible for this activity, in that previous studies of the POU domain have shown that it has a very low affinity for DNA (9,15,20). The transcriptional activity of Oct-3 V-P was dependent on the presence of an intact octamer binding sequence in the target promoter. We therefore hypothesized that the octamer-dependent activity of Oct-3 V-P was conferred by interaction of Oct-3 V-P with Oct-1 in the transfected HeLa cells. Oct-1, by itself, is unable to activate transcription of the octamer-containing reporter construct used in our experiments (42; Fig. 4C). In this model, therefore, we propose that Oct-1, while bound to an octamer motif in a promoter, can recruit Oct-3 to the promoter, thereby allowing the complex to strongly activate transcription using the Oct-3 C-terminal activation domain.

We present several independent lines of evidence that support the possibility of a functional interaction between Oct-1 and Oct-3. First, we demonstrated that Oct-3 can interact in vitro with the Oct-1 POU domain fused to GST. These in vitro reactions were performed in the presence of ethidium bromide, which has been shown to discriminate between true protein–protein interactions and apparent interactions which are dependent upon contaminating DNA in the assay (9,48). Second, in vivo transfection experiments, expression of either the Oct-1 or the Oct-3 POU domains abolished the ability of Oct-3 V-P to activate transcription. Our interpretation of these results is that the isolated POU domains acted in a dominant negative fashion to block the interaction of Oct-1 and Oct-3 V-P. Third, the variant Oct-3 V-P construct, lacking the POU domain, failed to transactivate the octamer-containing reporter gene in transfection experiments (Fig. 4C). These data would therefore suggest that the putative in vivo interaction between Oct-1 and Oct-3 required interactions involving the POU domains of the two proteins. Finally, a fusion protein between the Oct-3 POU domain and the VP16 acidic transactivation domain activated transcription in an octamer-dependent fashion. Again, the most plausible interpretation of this experiment is that the fusion protein is interacting with endogenous Oct-1 bound to octamer motifs in the reporter plasmid. This postulated Oct-1–Oct-3 interaction may have been driven, in our experiments, by the high levels of expression achievable in transient transfection assays. Nevertheless, it may be important to keep these results in mind when interpreting the action of POU factors in more natural settings. In particular, POU factors which, like Oct-3, are expressed in a developmentally regulated fashion may interact with the ubiquitously expressed Oct-1 and modulate transcription through octamer motifs. Future experiments will be needed to evaluate the extent to which such interactions are relevant to the control of normal development.

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