Oct-4 Regulates Alternative Platelet-derived Growth Factor α Receptor Gene Promoter in Human Embryonal Carcinoma Cells*

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Expression of the platelet-derived growth factor α-receptor (PDGFlαR) gene is tightly controlled in mammalian embryogenesis. A well established model system to study human embryogenesis is the embryonal carcinoma cell line Tera2. We have shown previously that retinoic acid-differentiated Tera2 cells express two PDGFlαR transcripts of 6.4 kilobase pairs (kb) (encoding the full-length receptor) and 3.0 kb, respectively, whereas in contrast, undifferentiated Tera2 cells express PDGFlαR transcripts of 1.5 kb and 5.0 kb. Here we show that this switch in PDGFlαR expression pattern during differentiation of Tera2 cells results from alternative promoter use. In undifferentiated cells, a second promoter is used, which is located in intron 12 of the PDGFlαR gene. Functional analysis shows that this promoter contains a consensus octamer motif, which can be bound by the POU domain transcription factor Oct-4. Oct-4 is expressed in undifferentiated Tera2 cells but not in retinoic acid-induced differentiated cells. Mutation of the octamer motif decreases promoter activity, while ectopic expression of Oct-4 in differentiated Tera2 cells specifically enhances the activity of this PDGFlαR promoter. Therefore, we suggest that an important aspect in the maintenance of the undifferentiated state of human embryonal carcinoma cells results from Oct-4 expression, which thereupon activates this PDGFlαR promoter.

Platelet-derived growth factor (PDGF) and its receptors play a prominent role during early mammalian development. Already in the preimplantation embryo of the mouse, from the two-cell stage onwards to the blastocyst stage, the PDGF-A chain is expressed (1), while both this gene and the cognate PDGF α-receptor (PDGFlαR) gene are expressed in early postimplantation embryos (2). Murine embryonal carcinoma (EC) cells in culture secrete PDGF-AA (3) and express the PDGFlαR following differentiation by retinoic acid (RA) is accompanied by a shift in expression of PDGFlαR mRNA variants (11). Four human PDGFlαR transcripts have been identified as a result of a combination of alternative splicing and promoter use. Two PDGFlαR mRNA species of 1.5 and 5.0 kb, respectively, are expressed in early human embryonic cells, including the undifferentiated Tera2 EC cells. Studies on surgically removed testicular germ cell tumors have shown that the 1.5-kb PDGFlαR transcript can be used as a selective marker for carcinoma-in situ, seminoma, and undifferentiated nonseminomatous tumors in the human testis. In differentiated cells, including RA-differentiated Tera2 (Tera2 RA) cells, two other PDGFlαR transcripts of 6.4 kb, which encodes the functional full-length receptor, and of 3.0 kb, which potentially encodes a dominant negative isoform, have been identified. Aberrant expression of the full-length PDGFlαR receptor, encoded by the 6.4-kb transcript, has also been implicated in tumorigenesis, i.e. it is overexpressed in various tumors, including gliomas (12).

In a previous study we cloned and characterized the human PDGFlαR gene promoter (P1), which gives rise to the 6.4- and 3.0-kb transcripts. Activity of this P1 promoter can be stimulated strongly by RA and cAMP (13). Similar studies with respect to the mouse and rat PDGFlαR promoter have been published recently (14, 15). In the present study we have cloned and characterized the second PDGFlαR gene promoter (P2), which gives rise to the 1.5- and 5.0-kb transcripts in early embryonic cells. We show here that the P2 promoter, located in intron 12 of the PDGFlαR gene, is active in undifferentiated Tera2 cells and is controlled by the POU domain transcription factor Oct-4. Oct-4 expression is detected in Tera2 RA cells but not in Tera2 RA cells.

* Supported by Dutch Cancer Society Grant NUKC-9010.
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‡ The abbreviations used are: PDGF, platelet-derived growth factor; PDGFlαR and PDGFβR, platelet-derived growth factor-α and -β receptor, respectively; RA, retinoic acid; EC, embryonal carcinoma; kb, kilobase pair(s); kPGF, Kapoisi's fibroblast growth factor; EMSA, electrophoretic mobility shift assay.
MATERIALS AND METHODS

Cell Culture—Tera2 clone 13 (Tera2) cells were grown in a modification of minimal essential medium lacking nucleosides and deoxyribonucleosides, supplemented with 10% (v/v) fetal calf serum and 44 mm NaHCO3 in a 7.5% CO2 atmosphere at 37 °C. Differentiation of cells was induced by the addition of RA (5 μM) 16 h after the cells were seeded at low density (5 x 103 cells/cm2) and maintained at this medium for 7 days, prior to further analysis or transfection.

PDGFαR P2 Promoter Constructs—Nucleotide sequence analysis was performed using the Pharmacia T7 sequencing kit. PDGFαR P2 promoter constructs were generated by standard cloning procedures (16), using either restriction fragments or DNA fragments obtained by the polymerase chain reaction. Mutation of the octamer binding site was performed with the Altered Sites system kit (Promega), according to the manufacturer’s protocol. All fragments were inserted in the multiple cloning site of the pSLL4 luciferase reporter plasmid (13).

Transfection, Luciferase, and β-Galactosidase Assays—Promoter constructs were transiently transfected into either undifferentiated (Tera2 EC) or differentiated (Tera2 RA) cells using the calcium phosphate coprecipitation method (16). Luciferase activity was determined 48 h (Tera2 EC) or 72 h (Tera2 RA) post-transfection (Luciferase assay kit, Promega). The luciferase activity was corrected for transfection efficiency by measuring the β-galactosidase activity of a lacZ gene driven by an SV40-promoter of a cotransfected pCH110 plasmid (17).

Every experiment was done in duplicate and repeated at least twice with two batches of DNA.

RNA Analysis—Total RNA was isolated from undifferentiated (EC) or differentiated (7 days of RA treatment) Tera2 cells, using the isothiocyanate method (18). After poly(A)+ isolation, the mRNA was quantitated spectrophotometrically and subjected to 1% agarose gel electrophoresis in formamide. The amount and integrity of loaded mRNA was controlled by ethidium bromide staining, after which it was transferred to Hybond-N (Amersham Corp.). Hybridization and washing procedures were carried out as described (11).

A mouse Oct-4 cDNA probe (kindly provided by Dr. P. C. van der Vliet, University of Utrecht), was cloned in front of a luciferase reporter gene, transiently transfected into undifferentiated (Tera2 EC) or differentiated (Tera2 RA) cells using the calcium phosphate coprecipitation method (16). Luciferase activity was determined 48 h (Tera2 EC) or 72 h (Tera2 RA) post-transfection (Luciferase assay kit, Promega). The luciferase activity was corrected for transfection efficiency by measuring the β-galactosidase activity of a lacZ gene driven by an SV40-promoter of a cotransfected pCH110 plasmid (17).

Every experiment was done in duplicate and repeated at least twice with two batches of DNA.

RESULTS

Sequence Determination and Characterization of the P2 Promoter of the PDGFαR Gene—Two alternative PDGFαR transcripts, of 1.5 and 5.0 kb, respectively, are specifically expressed in the undifferentiated Tera2 embryonal carcinoma cells. Although the transcripts terminate differently as a result of alternative splicing, both transcripts initiate in intron 12 of the PDGFαR gene (11). In order to functionally characterize this putative promoter, which was designated P2 promoter, the region was cloned and sequenced (Fig. 1). The sequence upstream from the transcription initiation site lacks a TATA box, which is also the case for the PDGFαR promoter in human (13), mouse (14), and rat (15), and is also not extremely GC-rich. In the promoter region, several consensus binding sites for transcription factors could be detected, including AP1, AP2, and PEAS motifs (23). A consensus octamer binding site is located in the transcribed region, at positions +28 to +35.

We first set out to determine the functional relevance of these putative binding sites and of other regions required for activity of the PDGFαR promoter. It is of interest to note that the P2 promoter region is demethylated in both undifferentiated and RA-induced differentiated Tera2 cells. A series of progressive deletion mutants of the PDGFαR P2 promoter was cloned in front of a luciferase reporter gene, transiently transfected into undifferentiated Tera2 cells, and assayed for promoter activity (Fig. 2). The luciferase activity of the complete P2 promoter fragment of approximately 2.5 kb (clone −2500/+182) was comparable with that of the much smaller clone −668/+182, indicating that no important expression information is pertained upstream from nucleotide −668, up to exon 12 of the PDGFαR gene. A further deletion, down to position −102 (clone −102/+182), displayed only fractionally lower activity than the clones −2500/+182 and −668/+182, which further limits the region necessary for control of high level expression. The reverse orientation of the −668/+182 fragment in the pSLL4 vector (clone −668/+182R) almost completely abolished activity, demonstrating the orientation dependence of the P2 promoter. These data show that intron 12 of the PDGFαR gene contains a bona fide promoter.

Deletion mutants generated at the 3' end (clones: −668/+14; −102/+14), which still included an intact transcription initiation site, reduced activity 4–5 times, compared with the parental clones −668/+182 and −102/+182. Thus, a cis-element determining high promoter activity is located in the transcribed part, within the region +14 to +182. The consensus octamer motif ATGCTAAT at position +28 to +35, which is present in all the constructs that show high promoter activity, was therefore mutated to the sequence AGGCCTAT (clones −668/+182M and −102/+182M, respectively). This mutation

3 H. J. Kraft, unpublished results.
Oct-4 Regulates PDGFaR Promoter in Human EC Cells

is expected to abrogate all fortuitous binding of POU domain-specific proteins (24, 25). Upon changing these two nucleotides in the octamer motif, promoter activity drops by a factor of 3–4, comparable with deletion of the complete +14/+182 region (Fig. 2). This indicates that the octamer motif is indeed involved in directing P2 promoter activity in Tera2 EC cells.

In order to demonstrate that Tera2 EC nuclear proteins can actually bind to the octamer motif, an EMSA was performed. It is shown in Fig. 3A that the intact −102/+182 promoter fragment forms a complex with nuclear extracts of Tera2 EC cells. The formation of this complex can be specifically competed by excess (100 times) unlabeled probe itself, but not by the −102/+182M fragment containing the mutated octamer motif (lane 4). Moreover, the −102/+182M fragment does not form a complex in this EMSA (Fig. 3A, lanes 5–8). In addition, the −102/+182M fragment competed efficiently the four specific complexes of a consensus oct-1c oligonucleotide (Promega) with nuclear extracts of EC cells, while the −102/+182M fragment was refractory to competition (Fig. 3B).

In conclusion, an octamer motif is involved in the regulation of the P2 promoter of the PDGFaR gene in undifferentiated human embryonal carcinoma cells. Oct-4 Binds to the PDGFaR P2 Promoter Octamer Motif—As previously described, the major change in constitutents of octamer binding proteins during retinoic acid-induced differentiation of murine embryonal carcinoma cells involves the down-regulation of Oct-4 expression (26). We hence hypothesized that also in the undifferentiated human embryonal carcinoma Tera2 cells the POU domain transcription factor Oct-4 is present and occupies the promoter P2 octamer motif, oct-P.

In order to test this hypothesis a series of EMSAs were performed with a double-stranded oligonucleotide (oct-P), which results in a higher resolution compared with the long promoter fragment. The oct-P contains the octamer sequence and flanking 7 nucleotides at the 5′ side and 8 nucleotides at the 5′ side of the P2 promoter (+20/+42). The resulting complexes were compared with the complexes formed by a consensus oct-1c oligonucleotide (see above). The oct-P as well as the oct-1c oligonucleotide gave rise to the formation of several complexes with nuclear proteins of Tera2 EC cells, Tera2 RA cells, or mouse F9 EC cells, which could be specifically competed by excess of the respective cold probe itself (Fig. 4; see also Fig. 5). In the EMSAs, oct-1c and oct-P displayed identical bandshift patterns (not shown). Based upon the complexes formed with the nuclear extracts of mouse F9 EC cells (27, 28) a positive identification of the Oct-4 complex with Tera2 EC or RA extracts was made possible, and is indicated in Fig. 4A. This shows that also during RA-induced differentiation of human Tera2 embryonal carcinoma cells the POU domain transcription factor Oct-4 is down-regulated.

To confirm the results obtained with the above described EMSAs, a Northern blot analysis was performed on mRNA of Tera2 EC and Tera2 RA cells. The blot was probed with a labeled Oct-4 cDNA, which showed that Oct-4 mRNA is present in Tera2 EC cells, contrasting with the absence of any detectable Oct-4 mRNA in Tera2 RA cells (Fig. 4B). Conclusive evidence that the indicated Oct-4 complex in the EMSAs is formed with this transcription factor comes from a supershift analysis. This analysis was performed with an anti-Oct4-antibody (22), which only supershifted the Oct-4-containing complex and not the Oct-1 complex (Fig. 4C).

Hence, undifferentiated human Tera2 embryonal carcinoma cells express Oct-4, which can complex with the P2-octamer motif. The cells cease to express Oct-4 upon RA-induced differentiation, which necessarily excludes complex formation between Oct-4 and the oct-P motif.

Comparison of the oct-P Binding Site with the Consensus oct-1c Motif—The members of the POU transcription factor family are defined by their ability to bind to the octamer motif. This causes experimental pitfalls to distinguish between the binding of the different members to a particular motif in a specific cell type (29). In addition to the octamer consensus motif, however, the nucleotides juxtaposed to the motif are also important to the affinity and specificity of binding of a given Oct protein (24). Therefore, we set out to compare the binding properties of the consensus oct-1c and the PDGFaR gene-derived oct-P motifs with nuclear extracts from Tera2 EC cells in EMSAs. The oct-P-derived complexes could not be competed by a 500-fold excess of the consensus oct-1c oligonucleotide, while competition with oct-P itself was easily established. A 100-fold excess of oct-P competitor was sufficient for strong competition. No signal was detectable with a 500-fold excess of competitor, even upon prolonged exposure (Fig. 5). This indicates that the oct-P oligonucleotide is bound stronger by POU proteins from Tera2 cells than the consensus oct-1c oligonucleotide. The results with the oct-P oligonucleotide are confirmed by the reciprocal experiment, using the oct-1c oligonucleotide as probe. Even with 100-fold excess, the oct-P competitor abolished the specific binding to the oct-1c oligonucleotide, while the oct-1c oligonucleotide was needed in larger

* H. R. Schöler, personal communication.
**DISCUSSION**

Endogenous OEF-4 induces high levels of both PGD2 and COX-2 in human EC cells. The expression of OEF-4 increases the expression of both PGD2 and COX-2 in human EC cells. The effect of OEF-4 on the expression of PGD2 and COX-2 is shown in Figure 1. The expression of PGD2 was measured by real-time RT-PCR and Western blotting. The expression of COX-2 was measured by Western blotting. The expression of both PGD2 and COX-2 was significantly increased in human EC cells treated with OEF-4.

**Figure 1**

**A**: Expression of PGD2 and COX-2 in human EC cells treated with OEF-4. The expression of PGD2 and COX-2 was significantly increased in human EC cells treated with OEF-4. **B**: Activity of COX-2 in human EC cells treated with OEF-4. The activity of COX-2 was significantly increased in human EC cells treated with OEF-4.
**Fig. 4.** Transcription factor Oct-4 mRNA and protein is present in undifferentiated EC but not in RA-differentiated Tera2 cells. 

A, an EMSA was performed with nuclear extracts from undifferentiated (EC) or 7 days RA-differentiated (RA) Tera2 cells or undifferentiated mouse F9 embryonal carcinoma cells (F9) with the PDGFrα oct-P oligonucleotide. The EMSA was performed under the conditions indicated in the legend of Fig. 3. The identified complexes are depicted in the figure with arrows. F, free probe. B, Northern blot analysis with mRNA of undifferentiated EC or 7 days RA-differentiated Tera2 cells. The blot was hybridized with a mouse Oct-4 cDNA probe, washed at 55 °C in 0.1 × SSC and exposed for 2 days at -80 °C with intensifying screens. C, supershift analysis with a mouse anti-Oct-4-antibody (see Ref. 22). The PDGFrα promoter oligonucleotide oct-P was used in an EMSA with nuclear extracts of Tera2 EC cells. A mouse anti-Oct-4-antibody was used with increasing concentrations to perform a supershift. The Oct-4 and the supershifted complexes are indicated in the figure. Conditions are as described in the legend of Fig. 3. F, free probe.
Fig. 5. The octamer sequence of the PDGFα-R P2 promoter (oct-P) displays higher binding affinity toward POU domain proteins than the consensus oct-1c site. An EMSA was performed with nuclear extracts from Tera2 EC cells (lanes 2-6) and the PDGFα-R oct-P oligonucleotide as probe (P, free probe). The complexes were competed with different-fold excess of either the cold oct-P probe (P) or consensus oct-1c oligo (1c), as indicated.

differentiation of the EC cells reduces transcription of the gene (33), which is probably due to a decline in the level of Oct-4 (22). Apart from Oct-4-controlled expression, REX-1, kFGF, and alternative PDGFα-R transcripts contain no obvious denominator. This stresses the role of Oct-4 to stir diverse actions in development.

Oct-4, as well as any other POU domain transcription factor, is able to complex (in vitro) with the consensus octamer binding motif of the P2 promoter. The absence of P2-initiated messengers in RA-induced differentiated Tera2 cells or in normal placenta indicates that this motif is not used promiscuously by other POU domain transcription factors like e.g. Oct-1, and that the specificity of binding is probably highly influenced by the flanking sequences of the motif. A preliminary comparison
Oct-4 Regulates PDGFαR Promoter in Human EC Cells

of the octamer flanking sequences of kFGF, REX-I, and PDGFαR shows, however, that the motifs cannot be aligned adequately to explain the preferential binding of Oct-4 to these sites. Accessory proteins might therefore explain Oct-4-dependent regulation (see above).

The role of proteins encoded by these alternative PDGFαR transcripts, if any, remains obscure, since presently none of these proteins have been detected in vivo. The sequence of the 5.0-kb messenger suggests a putative oncogene-like action, which may be important for autonomous growth of Tera2 EC cells. Undifferentiated Tera2 cells have been shown to proliferate in the absence of serum growth factors (34). A possible role in development or differentiation may also be inferred from the expression patterns of alternative PDGFαR transcripts. P2-initiated transcripts have been identified in human oocytes and preimplantation stages⁵ and in human testis tumors.² The down-regulation of the 1.5-kb transcript expression in spermatogenesis suggests that the P2-promoter is active in cells of the female primordial germ cell lineage, probably in the commitment of cells during embryonal development. Alternative transcripts, generated by differential promoter use and/or splicing, of other tyrosine kinase receptors have been described, including the genes for PDGFβR (4), FGFR receptors (35), epidermal growth factor receptor (36), c-kit (37) and PDGFαR of the mouse (38). Interestingly, an alternative transcript of approximately 4.8 kb of the PDGFαR gene has been detected in the mouse embryonal carcinoma cell line F9, but only after RA-induced differentiation (38). This transcript has also been described by Lee et al. (39) and is regulated in a differentiation-specific manner. Any conclusive evidence concerning functions of the corresponding alternative proteins remains lacking, however.

In conclusion, the POU transcription factor Oct-4 controls the developmentally regulated expression by the PDGFαR P2 promoter. Whether the down-regulation of Oct-4 during RA-induced differentiation is a prerequisite for the activation of the PDGFαR promoter P1 remains to be determined. We are currently working toward this goal.

Acknowledgments—We appreciate the work of Evert-Jan Overdijk in cloning and sequencing parts of the P2 promoter. We are grateful to Drs. L. H. J. Looijenga, J. W. Oosterhuis, H. R. Schijler, M. J. T. van Eyck, J. Mandelbaum, and C. L. Mummery for helpful discussions and communication of unpublished results. We thank Dr. P. C. van der Vliet for supplying the CMV-Oct-4 expression vector.

REFERENCES