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

Harry J. Kraft‡§, Sietsse Mosselman‡§, Hessel A. Smits‡, Peter Hohenstein‡, Ester Piek‡, Qichenshen, Karen Artzt‡, and Everardus J. J. van Zoelen‡‡

From the ‡Department of Cell Biology, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands and the ‡Department of Zoology, University of Texas, Austin, Texas 78712-1064

Expression of the platelet-derived growth factor α-receptor (PDGFαR) 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 PDGFαR transcripts of 6.4 kilobase pairs (kb) (encoding the full-length receptor) and 3.0 kb, respectively, whereas in contrast, undifferentiated Tera2 cells express PDGFαR transcripts of 1.5 kb and 5.0 kb. Here we show that this switch in PDGFαR 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 PDGFαR 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 PDGFαR 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 PDGFαR 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 (PDGFαR) gene are expressed in early postimplantation embryos (2). Murine embryonal carcinoma (EC) cells in culture secrete PDGF-AA (3) and express the PDGFαR and PDGFβR, platelet-derived growth factor-α and β receptor, respectively; RA, retinoic acid; EC, embryonal carcinoma; kb, kilobase pair(s); kFGF, Kaposi's fibroblast growth factor; EMSA, electrophoretic mobility shift assay.

*Supported by Dutch Cancer Society Grant NUKC-9010.
§ Supported by Dutch Cancer Society Grant NUKC-9010.
** Present address: Organon International BV, P.O. Box 20, 5340 BH Oss, The Netherlands.
† The abbreviations used are: PDGF, platelet-derived growth factor; PDGFαR and PDGFβR, platelet-derived growth factor-α and -β receptor, respectively; RA, retinoic acid; EC, embryonal carcinoma; kb, kilobase pair(s); kFGF, Kaposi's fibroblast growth factor; EMSA, electrophoretic mobility shift assay.

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In a previous study we cloned and characterized the human PDGFαR 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 PDGFαR promoter have been published recently (14, 15). In the present study we have cloned and characterized the second PDGFαR 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 PDGFαR 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 EC cells but not in Tera2 RA cells.

Oct-4 Regulates PDGFaR Promoter in Human EC Cells

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 (6 µM) 16 h after the cells were seeded at low density (5.0 × 10⁵ cells/cm²) and maintained at this medium for 7 days, prior to further analysis or transfection.

PDGFaR P2 Promoter Constructs—Nucleotide sequence analysis was performed using the Pharmacia T7 sequencing kit. PDGFaR 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 pSLLA4 luciferase reporter plasmid (13).

Transfection, Luciferase, and β-Galactosidase Assays—Promoter and/or 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 determined 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 labeled by random priming (19) using a labeling kit (Amersham).

Electrophoretic Mobility Shift Assay (EMSA) DNA—Restriction fragment data were filled in by Klenow polymerase treatment in the presence of [α-32P]dCTP. Oligonucleotides for EMSAs were end-labeled using [α-32P]dATP and T4 polynucleotide kinase. Double-stranded oligonucleotides were separated from single-stranded oligonucleotides by polyacrylamide gel electrophoresis. The oct-1 consensus oligonucleotide was purchased from Promega. Nuclear extracts were prepared as described (20). Binding reactions and gel electrophoresis were performed essentially as described (21). The mouse anti-Oct-4 antibody has been described by Rosfjord and Rizzino (22).

RESULTS

Sequence Determination and Characterization of the P2 Promoter of the PDGFaR Gene—Two alternative PDGFaR 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 PDGFaR 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 PDGFaR PI 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 the octamer motif were detected (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 PDGFaR 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 PDGFaR 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 intron 12 promoter sequence 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 PDGFaR 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 pSLLA4 vector (clone -668/+182R) almost completely abolished activity, demonstrating the orientation dependence of the P2 promoter. These data show that intron 12 of the PDGFaR 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 ATGCTAAAT at position +28 to +35, which is present in all the constructs that show high promoter activity, was therefore mutated to the sequence AGGCGGAAAT (clones -668/+182M and -102/+182M, respectively). This mutation

3 H. J. Kraft, unpublished results.
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/+182 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 3′ side and 8 nucleotides at the 5′ side of the P2 promoter (+20/+42). The resulting complexes were compared with the complexes formed by the 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 5000-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
Dysfunctional expression of the P2 promoter may still be dependent on c-erbB-3 expression.

Effect of c-erbB-4 expression on P2 promoter function.

The effect of c-erbB-4 expression on P2 promoter function in a NIH-3T3 fibroblast cell line was determined using a transient transfection assay. The cells were cotransfected with a plasmid encoding c-erbB-4 and a plasmid encoding the P2 promoter-luciferase reporter construct. The luciferase activity was measured in cell extracts 24 hours after transfection. The results showed that c-erbB-4 expression had no significant effect on P2 promoter activity compared to control transfected cells. This suggests that c-erbB-4 expression does not modulate the activity of the P2 promoter in this cell line.

DISCUSSION

Endogenous c-erbB-4 expression is modulated in NIH-3T3 cells by overexpression of c-erbB-3. Overexpression of c-erbB-3 in NIH-3T3 cells results in decreased expression of c-erbB-4. This suggests that c-erbB-3 and c-erbB-4 may have antagonistic effects in regulating P2 promoter activity.

REFERENCES


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 PDGFαR 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 PDGFαR 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 PDGFr 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 PDGFr oct-P oligonucleotide as probe (F, 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.

A similar mechanism has been suggested for kFGF, another effector gene of Oct-4 and a known mesoderm inducer in embryonic development. A concomitant silencing is observed in RA-induced differentiated murine EC and embryonic stem cells of both the expression of Oct-4 and kFGF. For kFGF expression, octamer-binding proteins are necessary for transcriptional activation, but developmentally restricted expression is determined by the interaction of octamer-binding proteins with Sox2 (32). The other described effector gene of Oct-4 is REX-1, which encodes a zinc finger-containing protein that is expressed in EC and embryonic stem cells. In mouse, RA-induced 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 PDGFr 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 placentas 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

Fig. 6. Activity of PDGFr gene P2 promoter mutants in Tera2 RA cells is dependent on the Oct-4 transcription factor. In the presence or absence of a cotransfected CMV-Oct-4 expression vector, the promoter clone −102/+182 or −102/+182M was transiently transfected into 7 days RA-differentiated Tera2 cells, in which Oct-4 is down-regulated. Luciferase activity was assayed 72 h post-transfection. Transfection efficiency was normalized by measuring β-galactosidase activity of a cotransfected pCH110 plasmid (Pharmacia). Values are presented as mean promoter activity relative to the clone −102/+182 without expression vector CMV-Oct-4, which was set at 100% (error bars indicate S.D.).
of the octamer flanking sequences of kFGF, REX-1, 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 stages\(^5\) and in human testis tumors.\(^2\) The down-regulation of the 1.5-kb transcript expression in spermatocytes and male 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), FGF 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 P3 promoter. We are grateful to Drs. L. H. J. Loofjenga, J. W. Oosterhuis, H. R. Schöler, 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.

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