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The Biological Function of a Fragment of the Neurotrophic Factor from Pigment Epithelium: Structural and Functional Homology with the Differentiation Factor of the HL-60 Cell Line

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Abstract—It was shown that the full-size neurotrophic factor from pigment epithelium (PEDF) induces the cell differentiation of the human promyelocyte leukemia cell line HL-60. A structural analysis of PEDF revealed in its *C*-terminal region a six-membered peptide fragment PEDF-(352–357) (PEDF-6) whose sequence is highly homologous to the 41–46 fragment of the active site of the human leukocyte differentiation factor HLDF (HLDF-6). The biological effect of PEDF and synthetic peptides PEDF-6 and HLDF-6 on the HL-60 cells and the early gastrula ectoderm of *Xenopus laevis* embryos was studied. On the basis of the structural and functional homologies of HLDF, PEDF, and their homologous peptides and the computer models of the spatial structures of the full-size PEDF and the PEDF with the *C*-terminal fragment split off tby the cleavage of the Leu^{380}–Thr^{381} bond in the serpin loop, a hypothesis on the functional role of the serpin loop in PEDF was put forward.

Key words: cell differentiation; cell differentiation factors; peptides, computer modeling

INTRODUCTION

Differentiation factors are substances that induce the transformation of primary (undifferentiated) cells into mature (functionally active) cells.[2] These factors can stop the unlimited tumor proliferation and induce differentiation of tumor cells. Most of endogenous differentiation factors are of protein nature; however, known are also some low-molecular-mass factors, such as retinoic acid [1, 2] and vitamin D_3 [3].

We have isolated HLDF from a culture of the HL-60 cell line of human promyelocyte leukemia induced by *E*-retinoic acid [4]. This is a small 54-aa glycosylated protein with *M* 8.2 kDa. HLDF can induce differentiation of HL-60 cells into phenotypically mature granulocytes [4]. In the *C*-terminal part of its polypeptide chain, we found a 6-membered peptide fragment (HLDF-6) that completely reproduced the differentiating activity of the native factor [5].

A computer-aided comparative analysis of the amino acid sequence of HLDF and currently available primary protein structures revealed that one more differentiation factor, PEDF from a cell culture of the human retinal pigment epithelium, contains a 17-aa *C*-terminal region homologous to the *C*-terminal fragment of HLDF (53% homology) [6]. PEDF is a neurotrophic factor that induces differentiation of the Y79 retinoblastoma cells into mature neurons [7], substantially decreases the rate of apoptosis of these cells [8], and protects them from the toxic action of some compounds, e.g., glutamic acid [9, 10]. PEDF has been detected in almost all mammalian and avian nervous tissues [11] and in many other tissues, e.g., in lung epithelium [12].

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[2] Abbreviations: BSA, bovine serum albumin; EGE, early gastrula ectoderm of *Xenopus laevis* embryos; HLDF, differentiation factor from the HL-60 cell line of human promyelocyte leukemia; NBT, Nitro Blue Tetrazolium dye; PBS, phosphate-buffered saline; and PEDF, pigment epithelium-derived differentiation factor.

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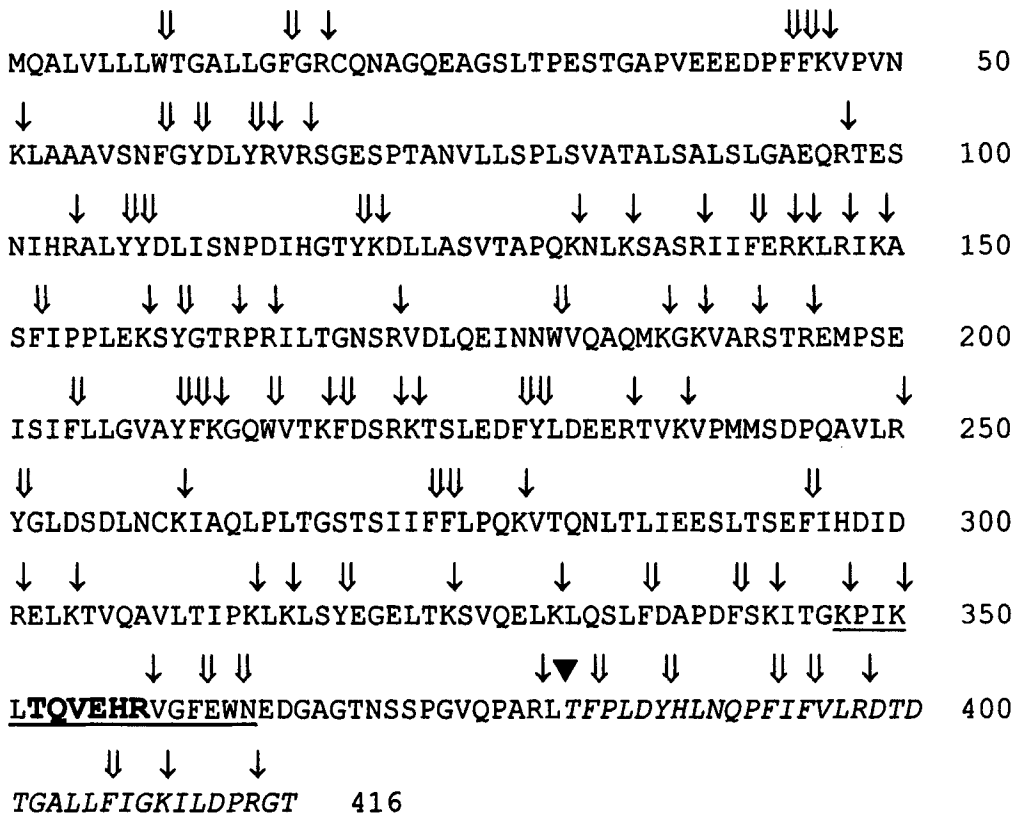

Fig. 1. Amino acid sequence of bovine PEDF [6]. Bonds cleaved by trypsin (↓) and chymotrypsin (⇓) and the L^{380}–T^{381} bond in the central part of the serpin loop, which is highly sensitive to proteolysis (▼), are indicated. The *C*-terminal fragment (381–416), removed upon the cleavage of this bond, is italicized. The region homologous to the *C*-terminal fragment of HLDF (HLDF-like loop) is underlined. The PEDF-6 fragment is shown in bold-face type.

The PEDF molecule consists of 418 aa and has $M \sim 50$ kDa (Fig. 1) [6]. On the basis of the primary and spatial structures, PEDF was assigned to the family of serpins (serine protease inhibitors) [6], although it displayed no inhibitory activity. Like other serpins, PEDF has a globular structure with an extruding loop containing a bond highly sensitive to serine proteases [13]. The cleavage of this bond results in the splitting off of a 36-membered *C*-terminal fragment and some conformational changes in the whole PEDF molecule, which follows from a decrease in its molecular ellipticity [14]. These changes do not however affect the activity of PEDF toward either retinoblastoma cells or mature neurons. The regulatory role of the cleavable *C*-terminal fragment of PEDF remains therefore unclear.

The goal of this work was a comparative study of the action of PEDF, HLDF, and biologically active hexapeptides PEDF-6 and HLDF-6 on the HL-60 cells and EGE in order to determine the functional role of the PEDF *C*-terminal fragment.

RESULTS AND DISCUSSION

The primary structures of homologous *C*-terminal parts of HLDF and PEDF are given in Table 1. Previously, we found that it is the *C*-terminal fragment of HLDF containing the HLDF-6 six-membered sequence that is responsible for the differentiating activity of the whole HLDF molecule [5, 15]. It was therefore logical to assume that PEDF, which contains a homologous site, can also induce differentiation of HL-60 cells.

An examination of the differentiating effect of PEDF on the HL-60 cells using the NBT-test indicated that this factor is a highly effective inducer of differentiation of the human promyelocyte leukemia cells: it is active in a rather broad concentration range, from 10^{-7} to 10^{-10} M (Table 2).

The similarity in the effects of PEDF and HLDF on the HL-60 cells suggested that the differentiating activity of PEDF is due to its 352–357 fragment TQVEHR (PEDF-6), which is homologous to fragment TGENHR (41–46), the active site of HLDF (HLDF-6).

To test this hypothesis, PEDF was subjected to the exhaustive hydrolysis by a mixture of trypsin and chymotrypsin, and the resulting products were analyzed in the NBT-test on the HL-60 cells. Hydrolyzates of two other noninhibitory serpins, ovalbumin and BSA, served as controls (Table 3). The sequencing of PEDF indicated that, upon exhaustive trypsin–chymotrypsin hydrolysis, none of the bonds in the PEDF-6 fragment

was cleaved, so that it should be present in the hydrolyzate as part of fragment 351–357 LTQVEHR (Fig. 1).

The fact that the PEDF hydrolyzate retained the differentiating activity favored our assumption that TQVEHR, the 352–357 segment of PEDF, can act as a differentiation inducer of the HL-60 cells. To conclusively confirm this assumption, we synthesized PEDF-6 and examined it in the NBT-test. As the results presented in Table 2 show, this peptide fully reproduces the differentiating activity of the full-size PEDF on the HL-60 cells.

We studied the differentiating activity of PEDF and synthetic PEDF-6 on EGE, the embryonic tissue most sensitive to various differentiation factors, and compared their action with the effect of HLDF and HLDF-6, its active fragment. Both HLDF and HLDF-6 induced the blood cell formation in EGE (Figs. 2a, 2b). The full-size PEDF induced mainly the EGE differentiation by the neural type: eye tissues, retina and pigment epithelium (Fig. 2c), or the neural tube (Fig. 2d) were first differentiated, although some blood cells were also formed in this case. On the other hand, synthetic PEDF-6 induced the EGE differentiation in the mesenchymal cells (Fig. 2e). Neither blood cells nor neural structures were formed in EGE treated with PEDF-6.

In control EGE explants, which were not treated with the inducer, no differentiation was observed; only cells of atypical epidermis were seen on the slices (Fig. 2f).

Since the full-size PEDF has a twofold effect on the EGE (it induces the formation of both neural structures and blood cells), it would be logical to assume that the neural differentiation of EGE occurs through the action of its *N*-terminal fragment, which is known to induce the differentiation of retinoblastoma cells [13], whereas the PEDF-6 fragment induces the blood cell formation. However, the synthetic peptide with the PEDF-6 sequence induced the formation of mesenchymal rather than blood cells. We believe that, although the homologous sites PEDF-(352–357) and HLDF-(41–46) differ in their amino acid sequences, the functional groups of the PEDF-6 fragment in the molecule of full-size PEDF acquire a conformation similar to that of the corresponding fragment in the active site of HLDF.

Surprisingly, we found that a PEDF preparation exposed to several freezing–thawing procedures gradually lost its differentiating activity toward both HL-60 cells and EGE. The analysis of this inactive preparation by SDS-PAGE showed that a band corresponding to a 46-kDa protein appears (data not shown) instead of the band corresponding to the 50-kDa full-size PEDF. This indicated that a limited proteolysis of the PEDF occurs during the freezing/thawing. The *N*-terminal sequencing of the PEDF preparation subjected to several freezing/thawing cycles revealed only the TFPLDYHLNQPFIFVLRDT sequence. As the *N*-terminal Met residue of PEDF is acetylated, the appearance of the sequence identical to that of fragment 381–416 of

Table 1. Primary structures of homologous *C*-terminal sites of differentiation factors PEDF [6] and HLDF [4]*

PEDF	347	KP I KL<u>TQVE-HR</u>AG-F E WN*	363
HLDF	37	KE L-L<u>TG-ENHR</u>C G I F V I N	53

* Residues coinciding in the two factors are shown in bold-face type; fragments PEDF-6 and HLDF-6 are underlined.

Table 2. Differentiating activity of PEDF and peptide TQVEHR, corresponding to the PEDF-6 fragment, at various concentrations*

Protein/Peptide concentration, M	PEDF	Peptide TQVEHR
10^{-6}		56 ± 1
10^{-7}	56 ± 2	52 ± 1
10^{-8}	55 ± 1	48 ± 1
10^{-9}	49 ± 1	39 ± 1
10^{-10}	33 ± 1	30 ± 1
Control	28 ± 3	

* The amount of NBT-positive cells (%) is given.

Table 3. Differentiating activity of hydrolyzates of PEDF, ovalbumin, and BSA at various concentrations. For the exhaustive hydrolysis, a mixture of trypsin and chymotrypsin was used

Concentration, M	NBT-positive cells, %		
	PEDF	ovalbumin	BSA
10^{-8}	54 ± 2	26 ± 2	34 ± 2
10^{-9}	49 ± 1	29 ± 2	29 ± 2
10^{-10}	33 ± 1	29 ± 2	29 ± 2
Control (a mixture of trypsin and chymotrypsin)		29 ± 5	

PEDF indicates that, during the freezing/thawing, a single bond, L^{380}–T^{381}, is cleaved (Fig. 1). It is known that the L^{380}–T^{381} bond is located in the middle of the serpin loop and is highly sensitive to serine proteases. During its hydrolysis, a 5-kDa decrease in the molecular mass of the protein usually occurs [13], which is similar to that observed in our experiment. The cleavage of this bond does not affect the differentiating activity of PEDF on the retinoblastoma cells but leads to a loss of this activity on HL-60 cells and EGE.

It was previously found by CD that the cleavage of the L^{380}–T^{381} bond in recombinant PEDF changes the general ellipticity of the protein molecule, indicating some conformational changes of the overall structure [14]. We assumed that these changes involve the *C*-terminal part of the molecule adjacent to the serpin loop where the TQVEHR site (352–357) is located.

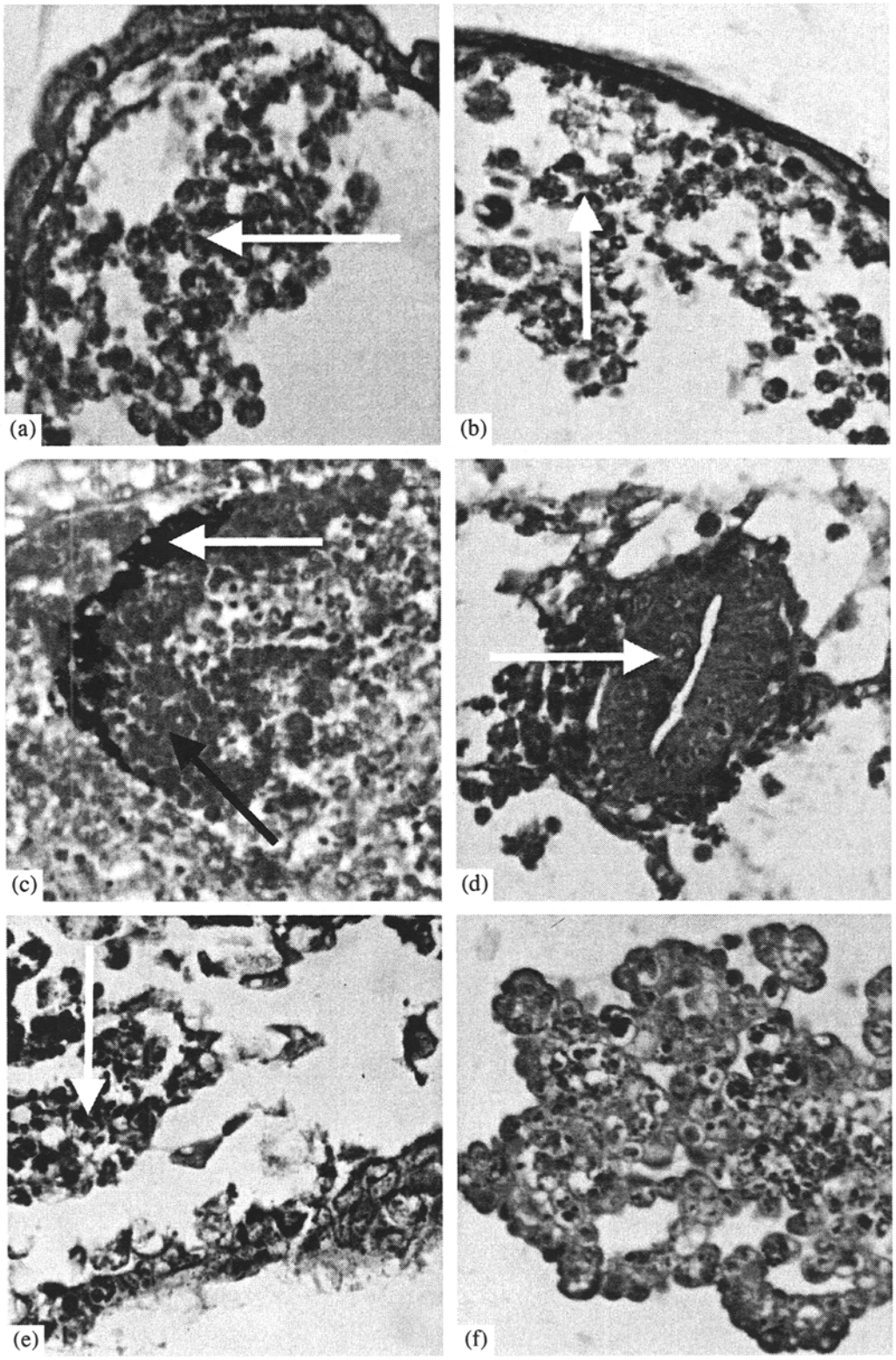

Fig. 2. Histological slices of explants of early gastrula ectoderm from *X. laevis* treated with various inducers: (a) blood cells induced by HLDF (shown with an arrow); (b) blood cells induced by HLDF-6 (shown with an arrow); (c), retina (solid arrow) and pigment epithelium (open arrow) induced by PEDF; (d), neural tube (shown with an arrow) and mesenchymal cells induced by PEDF; (e), treatment of EGE by the PEDF-6 peptide leads to the differentiation of mesenchymal cells only (shown with an arrow); and (f) the ectoderm that was not treated by inducers forms only atypical epidermis. The photographs were taken at a magnification of 10×25.

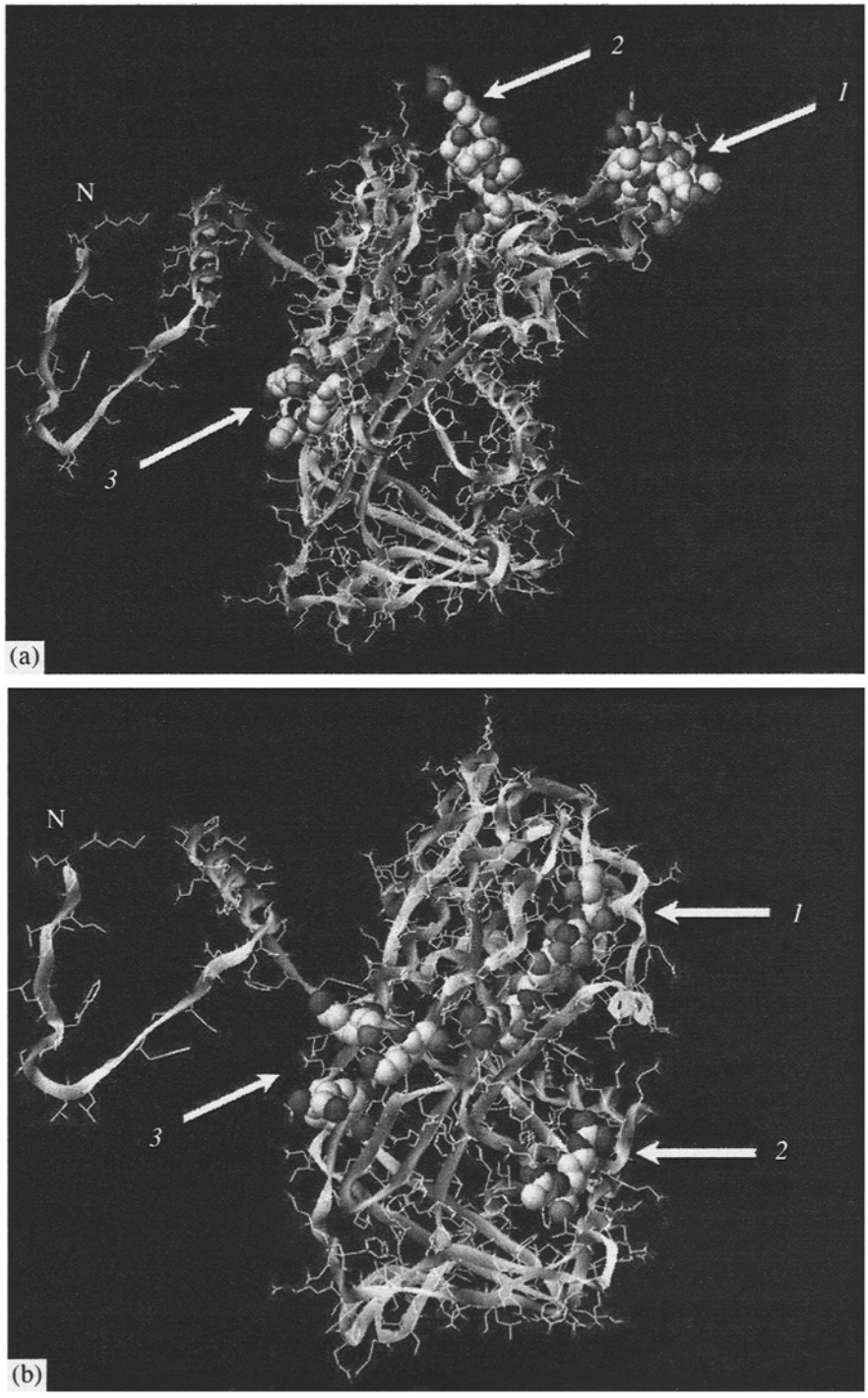

Fig. 3. Models of the 3D structure of the bovine PEDF proposed in this study: (a), full-size molecule; (b), a molecule truncated at the *C*-terminus due to the cleavage of the L^{380}–T^{381} bond in the serpin loop. (*1*) TQVEHR fragment (residues 352–357); (*2*) serpin loop (376–384) (a) and a portion of the serpin loop that remains upon truncation (376–380) (b); (*3*) heparin-binding Lys/Arg cluster (residues K^{132}, K^{135}, R^{147}, K^{187}, R^{192}, K^{212}); N, the *N*-terminus of the molecule.

To verify this, we constructed computer models of the 3D structures of the full-size bovine PEDF molecule (Fig. 3a) and the molecule that lacks the 36-aa *C*-terminal fragment, which is removed upon the cleavage of the L^{380}–T^{381} bond (Fig. 3b). The models are based on the homologies to the proteins with known 3D structures determined by X-ray analysis: antithrombin [17], antichymotrypsin [18], and the leukocyte elastase inhibitor [19]. These proteins belong to the family of serpins, and their homologies to bovine PEDF are 23.8,

23.3, and 23.1%, respectively. The model of PEDF without the 36-aa *C*-terminal fragment was constructed on the basis of the homologies to the same proteins truncated due to the break of the corresponding bonds in their serpin loops.

Both models agree well with the computer model of PEDF previously reported by Alberdi *et al.* [20]. According to our models and the Alberdi model, residues K^{132}, K^{135}, R^{147}, K^{187}, R^{192}, and K^{212} in the heparin-binding Lys/Arg cluster of PEDF are brought sterically close together (Figs. 3a, 3b).

Near the serpin loop (residues 376–384) of the full-size molecule (Fig. 3a, *2*), another loop (340–368), consisting of 29 aa, is situated somewhat farther from the *C*-terminus. Its amino acid sequence is homologous to that of the *C*-terminal fragment of HLDF, and PEDF-6 fragment is in the central part of this loop ("HLDF-like loop") (Fig. 3a, *1*). Interestingly, in our model of the molecule devoid of the *C*-terminal fragment, the corresponding region of the polypeptide chain is buried inside the globule (Fig. 3b, *1*) and, therefore, cannot function as a differentiation inducer. This is the reason for the loss by PEDF of its differentiating activity after the cleavage of the L^{380}–T^{381} bond in the serpin loop.

The role of the serpin loop and the cleavable *C*-terminal fragment of PEDF consists in maintaining the necessary conformation of the HLDF-like loop. It is likely that the *in vivo* cleavage of the serpin loop is one of the regulatory mechanisms providing for the qualitatively different activities of PEDF toward cells at different stages of their development.

The experiments on EGE suggest that the HLDF-like loop with its active fragment TQVEHR is responsible for the twofold function of PEDF on the embryonic cells: on the one hand, it induces the differentiation into blood cells, and on the other, it serves as an enhancer of the neural differentiation upon the action of the *N*-terminal fragment. PEDF interacts with EGE in a more complicated manner than with tumor cells, because EGE contains cells at the various stages of development.

The effect of various sites of the PEDF and HLDF molecules on the embryonic tissues will be studied further.

EXPERIMENTAL

The following preparations were used: RPMI 1640 and fetal calf serum from Gibco (United States); trypsin treated with *L*-1-tosylamido-2-phenylethyl-chloromethylketone and chymotrypsin from bovine pancreas treated with 1-chloro-3-tosylamido-7-amino-2-heptanone from Worthington (United States); ovalbumin, BSA fraction V, and Azocarmine from Serva (Germany); and chorionic gonadotropin (Russia).

Native PEDF was isolated from the vitreous body of the bovine eye as described in [21]. Distilled water was additionally purified using a Milli-Q system (Millipore, United States).

All other reagents were of the special purity grade.

Culturing of HL-60 cells. The cell line was kindly provided by R.G. Vasilov (Institute of Biotechnology, Moscow). Cells were grown in RPMI-1640 medium containing 7% fetal calf serum in an atmosphere of 5% CO_2 at 37°C.

NBT-test. The differentiating activity of factors and peptides was determined by the ability of HL-60 cells to reduce NBT [16]. Seventy-two hours after the addition of an inducer, the percentage of the NBT-positive cells was determined. Cells were defined as NBT-positive if they contained no less than 10 granules.

Exhaustive hydrolysis of proteins with a mixture of trypsin and α-chymotrypsin was performed for 12 h at 37°C in a 20 mM ammonium bicarbonate buffer, pH 7.8. The S : E ratio for each enzyme was 20 : 1. The enzymes were preliminarily tested using BSA as a substrate under the same conditions followed by SDS-PAGE of the hydrolysis products.

Peptide synthesis was performed by the solid phase method using the Boc/Bzl methodology on the polystyrene resin Boc-Arg(Tos)-PAM RESIN (100-200 mesh, 0.47 mmol/g; Advanced ChemTech, United States). For the synthesis of each peptide, 200 mmol of the starting amino acid was taken. Peptidylpolymers were synthesized in a flow-through reactor of variable volume using swellographic monitoring for the determination of the duration of operation cycles [22]. The resulting peptides were purified by gel filtration on Sephadex G-50 SF (column 26 × 90 mm) in 1 M AcOH and by subsequent medium-pressure liquid chromatography on a PepRPC™ column (FPLC-System; Pharmacia, Sweden). The purity of the preparations was determined by amino acid analysis and MALDI-TOF mass spectrometry. The mass-spectrometric analysis was carried out on a Vision 2000 instrument (Thermo Bioanalysis, UK).

Induction of EGE differentiation. The laying of spawn in spur-toed frogs *X. laevis* was artificially stimulated by intramuscular injections of chorionic gonadotropin. The spawn was cultured at 20°C to stage 10 according to the Nieuwkoop and Faber table [23]. The EGE was carefully cut out of the upper cover of embryonic blastocele [24]. The preparation of ectoderm, induction, and further culturing were carried out in a standard Niu–Twitty physiological saline [23].

The effective concentration of the inducer was 10^{-7} M (selected experimentally). The induction was performed for 1 h at 20°C. Then the ectoderm explants were transferred into a Niu–Twitty inducer-free solution and cultivated for 5 days at 20°C. The explants were then fixed with the Buen solution (15 : 5 : 1 picric acid–formalin–acetic acid) and subjected to a standard histological treatment: explants were embedded in paraffin, and 5-μm-thick slices were prepared and stained

successively with Azocarmine and the Mallori mixture [25]. The preparations were photographed using a Leica Dialux EB 22 light microscope.

Computer modeling of the spatial structure of PEDF. The 3D models were constructed on a computer (Silicon Graphics) using the Sybyl program, version 6.4 [26]. Sequences were aligned with the Needleman–Wunsch algorithm [27], which is supported by the COMPOSER module of the Sybyl 6.4 program. The significance of the alignment was determined from the alignment score criterion and checked by the jumbling procedure. The structural model of the bovine PEDF included 13 structurally conserved regions joined by 12 loops. A local correction of sterically disadvantageous contacts was performed followed by 300 steps of energy minimization using the Tripos force field.

SDS-PAGE of proteins was performed as described in [28] using linear 15% gels.

Protein concentration was determined by the Bradford method [29] using bovine serum albumin as a reference.

***N*-Terminal sequencing of proteins** was performed on an Applied Biosystems 447 sequencer (United States) according to the manufacturer's recommendations.

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