Expression of Platelet-Derived Endothelial Cell Growth Factor in *Escherichia coli* and Confirmation of Its Thymidine Phosphorylase Activity

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ABSTRACT: Platelet-derived endothelial cell growth factor (PD-ECGF) has been expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST). The fusion protein was purified by one-step affinity chromatography on glutathione–agarose beads, and recombinant PD-ECGF was proteolytically cleaved with thrombin from its GST leader peptide to yield pure protein. Recombinant PD-ECGF stimulated [^{3}H]methylthymidine uptake by endothelial cells in vitro; however, we were unable to detect stimulation of cell proliferation under a wide variety of conditions. We confirm that in accord with the recent report that PD-ECGF and human thymidine phosphorylase are products of the same gene [Furukawa, T., Yoshimura, A., Sumizawa, T., Haraguchi, M., & Akiyama, S. I. (1992) *Nature 356*, 668] recombinant PD-ECGF has thymidine phosphorylase activity comparable to that of *E. coli* thymidine phosphorylase. Further, *E. coli* thymidine phosphorylase was able to mimic the activity of recombinant PD-ECGF in the [^{3}H]methylthymidine uptake assay, and it appears that recombinant PD-ECGF's effect on the uptake of thymidine by endothelial cells may be due to modulation of cellular thymidine pools. The mechanism by which PD-ECGF stimulates angiogenesis remains to be elucidated.

Platelet-derived endothelial cell growth factor (PD-ECGF)[1] is a 45-kDa protein of low abundance, originally isolated as the sole endothelial cell mitogenic activity in platelets (Miyazono et al., 1987; Miyazono & Heldin, 1989). PD-ECGF is a nonglycosylated intracellular protein which lacks a secretion signal peptide and has no heparin binding activity (Ishikawa et al., 1989). It is posttranslationally modified by nucleotidylation and amino-terminal processing (Usuki et al., 1991). PD-ECGF is also chemotactic for endothelial cells in vitro (Ishikawa et al., 1989). Both the mitogenic activity and the chemotactic activity of PD-ECGF are reported to be largely restricted to endothelial cells, although activity on other cell types is now becoming apparent (Miyazono & Takaku, 1991). Transfection of PD-ECGF into a1-1 fibroblasts promotes growth of a more highly vascularized tumor when the cells are injected into nude mice (Ishikawa et al., 1989). Angiogenic activity of PD-ECGF has also been demonstrated on the chick chorioallantoic membrane (Ishikawa et al., 1989). Purification of PD-ECGF from human platelet lysates involves a six-step procedure (Miyazono et al., 1987; Miyazono & Takaku, 1991) with a yield of 30–40 μg from 800–1000 L of human blood or 40 μg of pure protein from one placenta (Usuki et al., 1990). In view of this exceptionally low abundance, we have expressed the PD-ECGF cDNA in *Escherichia coli* and report purification of 200 μg of pure soluble protein from 1 L of culture. The expression system employed the glutathione *S*-transferase (GST) fusion expression vector pGEX-2T (Smith & Johnson, 1988), which permits one-step affinity purification of the expressed protein. Proteolytic cleavage of the PD-ECGF from the GST leader peptide yields pure recombinant protein.

Recombinant PD-ECGF stimulated thymidine uptake in human and bovine endothelial cells. However, recombinant PD-ECGF did not stimulate the proliferation of these cells in vitro. The effect of PD-ECGF on thymidine uptake by endothelial cells may be the result of the molecules intrinsic thymidine phosphorylase activity.

MATERIALS AND METHODS

Materials

Bovine adrenal capillary endothelial (BACE) cells were isolated by clonal selection of primary cultures as described for their human counterparts (Fawcett et al., 1991). Human umbilical vein endothelial cells (HUVEC's) were isolated by collagenase digestion of perfused umbilical veins (Jaffe et al., 1973) and used between passages 1 and 4. Calf pulmonary artery endothelial (CPAE) cells CCL209 were from the American Type Culture Collection, Bethesda, MD. All cells were routinely cultured in 10% fetal calf serum/DMEM in a 5% carbon dioxide atmosphere. Culture media for BACE cells and HUVEC's were supplemented with heparin (90 μg/mL) and endothelial cell growth supplement (ECGS) (30 μg/mL). ECGS was prepared as described (Maciag et al., 1979). Heparin, collagenase type XI, glutathione–agarose, reduced glutathione, thrombin, thymidine, and *E. coli* thymidine phosphorylase were from Sigma. IPTG was purchased from Stratagene, and [^{3}H]methylthymidine (2 Ci/mmol) was from Amersham International, plc. Routine laboratory reagents were from BDH. All tissue culture media were prepared at the ICRF, Clare Hall Laboratories.

Methods

Construction of the Bacterial Expression Vector. Plasmid PL5, which contains the full-length PD-ECGF cDNA, was digested with *Apa*I and *Eco*RI, and the ends were filled using the 5′→3′ polymerase activity and 3′→5′ exonuclease activity of T4 DNA polymerase. *Apa*I restricts 33 base pairs upstream

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[1] Abbreviations: PD-ECGF, platelet-derived endothelial cell growth factor; HUVEC's, human umbilical vein endothelial cells; BACE, bovine adrenal capillary endothelium; CPAE, calf pulmonary artery endothelium; FCS, fetal calf serum; ECGS, endothelial cell growth supplement; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S*-transferase; SDS, sodium dodecyl sulfate.

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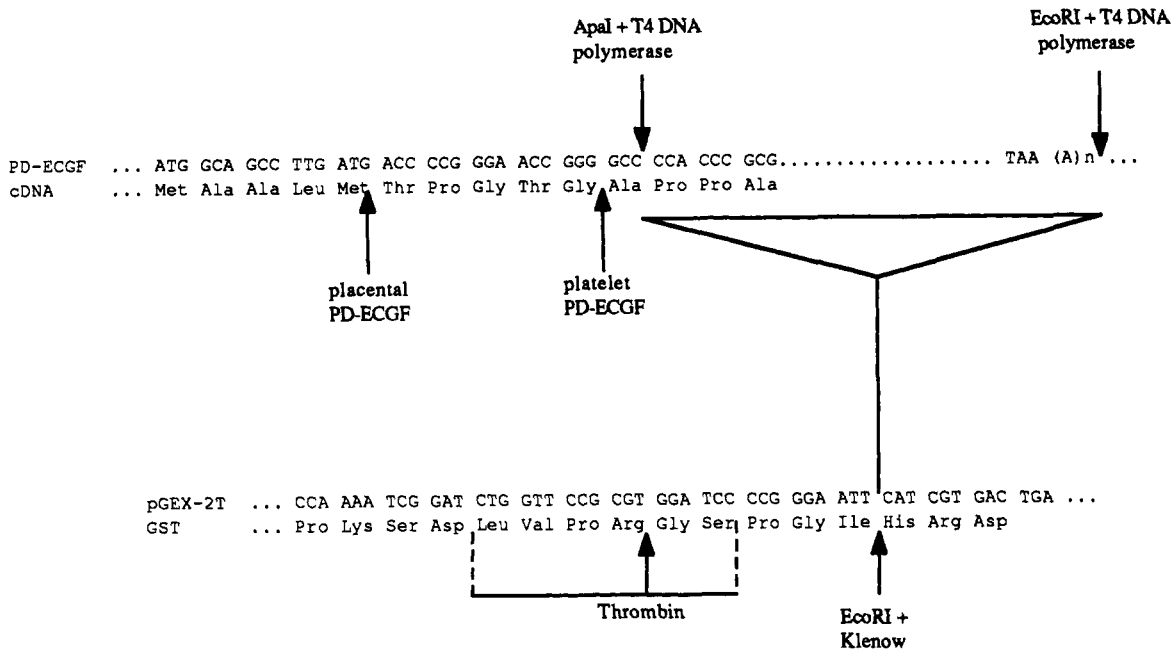

FIGURE 1: Construction of the GST–PD-ECGF expression vector (see text for details).

of the ATG initiation of translation site. The resulting DNA was ligated into the end-filled unique *Eco*RI site of plasmid pGEX-2T (Figure 1) to give plasmid pMOAL-10T. The construct was sequenced across the ligation junction to ensure a correct reading frame for the expression of GST–PD-ECGF fusion protein.

Expression and Purification of GST Fusion Proteins. GST fusion protein expression and purification were carried out as described (Smith & Johnson, 1988). Fresh overnight-saturated cultures of *E. coli* (strain TG1) transformed with either pGEX-2T or the recombinant pMOAL-10T were diluted 1:10 in L-broth containing ampicillin (100 μg/mL) and incubated at 37 °C with shaking. After 1 h of growth, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and cultures were incubated for a further 2 h at 37 °C or for 15 h at 15 °C. These were the optimal times for expression of the fusion protein at the noted temperatures. Analysis of total bacterial protein content was carried out by pelleting 1-mL aliquots in a microcentrifuge, boiling in sodium dodecyl sulfate (SDS) sample buffer (62 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 1% β-mercaptoethanol), and loading 10% of the sample onto an SDS–polyacrylamide gel. To compare the solubility of the expressed protein under different culture conditions, 1-mL bacterial cultures were centrifuged and resuspended in 100 μL of phosphate-buffered saline and lysed by sonication. The lysate was centrifuged at 10000*g* for 5 min and the supernatant removed. SDS sample buffer was added to both the supernatant and the pellet, the samples were boiled, and 10% was loaded onto SDS–polyacrylamide gels. Proteins were visualized by Coomassie blue staining. For affinity purification, bacterial cultures were pelleted by centrifugation at 5000*g* for 5 min at 4 °C and resuspended in 0.1 volume of 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 1% Triton X-100. The bacteria were lysed on ice by mild sonication and centrifuged at 10000*g* for 10 min at 4 °C. Glutathione–agarose slurry was added to bacterial supernatants (1–2 mL/L of broth), incubated for 10 min at 4 °C with shaking, and then poured into a column and washed with 10 column volumes of 50 mM Tris, pH 8.0, 150 mM NaCl, and 0.5 mM EDTA. Bound proteins were eluted in the same buffer containing 10 mM reduced glutathione. Alternatively, recombinant PD-ECGF was cleaved from the GST leader peptide by equilibrating the beads with 50 mM Tris, pH 7.5, 150 mM NaCl, and 2.5 mM $CaCl_2$ and incubating with thrombin (5–10 units/mg of fusion protein) for 12 h at 4 °C.

[^3H]Methylthymidine Uptake Assay. Low-passage HUVEC's were seeded in gelatin-coated 96-well plates at 10^4 cells per well and allowed to attach for 24 h in 10% FCS in Hepes-buffered DMEM. Medium was then removed and replaced with DMEM containing 10% FCS with or without growth factors. The cells were incubated at 37 °C for 48 h, [^{3}H]methylthymidine was added to 0.5 μCi/well, and the cells were incubated for a further 4 h. Cells were then washed with phosphate-buffered saline, removed with trypsin, harvested with a 96-well automated harvester onto printed glass fiber filters, dried, and counted in a flat-bed β-plate scintillation counter. Alternatively, growth factor and [^{3}H]methylthymidine were added to cells simultaneously 24 h after seeding and incubated for 48 h before harvesting and counting. CPAE and BACE were in turn seeded at 2000 cells per well in 10% FCS and allowed to quiesce for 10 days before adding the growth factors in 2% FCS. The cells were then processed as described for HUVEC's.

Cell Proliferation Assay. Endothelial cells were seeded in 6-well gelatin-coated tissue culture dishes at 50 000 cells per well in 10% FCS and allowed to quiesce for the same period as described above. The growth factors under test were added every 48 h in duplicate, at which point duplicates were also counted over a 10-day period.

Thymidine Phosphorylase Assay. Nucleoside phosphorylase assays were performed by spectrophotometric monitoring (Krenitsky et al., 1964). The assay solution was 10 mM thymidine in 0.1 mM sodium phosphate at pH 7.4 and an amount of enzyme to give a linear reaction rate (0.1–10 mg/mL). The mixture was incubated at 37 °C, and 0.8-mL aliquots were taken at 0, 5, 15, and 30 min, pipetted into 0.9 mL of 0.5 M sodium hydroxide, and read in a spectrophotometer at 300 nm. Correction was made for absorbance readings from that of a control incubation lacking enzyme. Correction was virtually constant throughout the incubation period. Concentrations of thymine were determined from a standard curve.

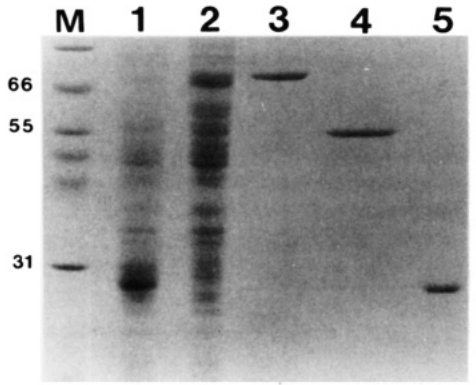

FIGURE 2: SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in a 12% polyacrylamide gel showing expression and purification of recombinant PD-ECGF in *E. coli*. Protein extract from 100 μL of culture of *E. coli* containing the parental vector pGEX-2T expressing GST (lane 1) or *E. coli* containing the recombinant pMOAL-10T expressing the GST–PD-ECGF fusion protein (lane 2). The fusion protein was purified on a glutathione–agarose column (lane 3), and recombinant PD-ECGF was proteolytically cleaved from the GST leader peptide while bound to the glutathione–agarose column and recovered from the supernatant (lane 4). The GST peptide remains bound to the affinity column (lane 5). Its purity confirms that the majority of the fusion protein has been processed by thrombin. Approximately 5 μg of each purified protein was loaded per lane. Molecular weight ($\times 10^{-3}$) markers are shown.

RESULTS

Expression and Purification of PD-ECGF. Expression and purification of PD-ECGF are shown in Figure 2. Total protein from 100 μL of broth containing bacteria transformed with the parental vector pGEX-2T (lane 1) or with the recombinant pMOAL-10T (lane 2) was resolved by SDS–polyacrylamide gel electrophoresis and visualized by Coomassie blue staining. Sonicates of the pMOAL-10T bacterial clone were prepared and passed over glutathione–agarose followed by extensive washing. Either the pure bound fusion protein was eluted with 10 mM reduced glutathione (lane 3) or the recombinant PD-ECGF was endoproteolytically cleaved by thrombin from its GST leader peptide while bound to the beads (lane 4). The cleaved but bound GST leader peptide was eluted by boiling in SDS-containing buffer (lane 5). Its purity confirms complete cleavage of the fusion protein. It is clear from the figure that the pMOAL-10T clone gives rise to a protein of 50 kDa (lane 4), consistent with the size of PD-ECGF predicted from the cDNA, and a fusion protein of 75 kDa (lane 3) where the GST leader peptide contributes a further 25 kDa (lane 5). The final PD-ECGF protein preparation was greater than 95% pure.

Effect of Fermentation Temperature on Solubility. Figure 3 respectively shows total soluble and insoluble proteins from 100 μL of culture of pMOAL-10T bacterial clones induced with IPTG at 37 °C for 2 h (lanes 1 and 2) or at 15 °C for 15 h (lanes 3 and 4). Sonicates were also prepared from 1 mL of broth induced at 37 °C (lane 5) and 15 °C (lane 6), and the soluble fraction was passed over 100 μL of glutathione–agarose slurry, washed, and eluted by boiling in an SDS-containing buffer. This figure shows that when the clones are induced at 37 °C, the majority of the induced protein is insoluble (<90%) while at 15 °C the fraction of soluble protein increases significantly. The yield of soluble protein is approximately 300 μg of fusion protein/L of culture and 200 μg of cleaved recombinant PD-ECGF protein/L of culture. A comparison of the purification and yields of PD-ECGF is given in Table I.

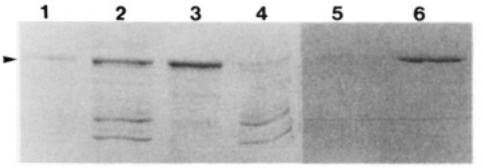

FIGURE 3: SDS–PAGE in a 12% polyacrylamide gel showing the solubility of the GST–PD-ECGF fusion protein when expressed at different temperatures. *E. coli* in the exponential phase of growth were induced with IPTG to express the fusion protein at 37 °C for 2 h (lanes 1 and 2) or at 15 °C for 12 h (lanes 3 and 4). Total soluble protein (lanes 1 and 3) and total insoluble protein (lanes 2 and 4) from 100 μL of broth were loaded onto gels. The fusion protein from the soluble fraction of 1 mL of culture was also purified from bacteria induced at 37 °C (lane 5) and 15 °C (lane 6) by affinity purification with glutathione–agarose and eluted in an SDS-containing buffer. The arrow indicates the position at which the GST–PD-ECGF fusion protein was expected to migrate.

Table I: Purification of PD-ECGF from Different Sources

source	quantity	no. of purification steps	quantity of PD-ECGF purified (μg)
platelets	1000 L of human blood	6	30–40
placenta	1	7	40
recombinant *E. coli*	1 L of culture	1	200

Table II: Comparison of the Amino-Terminal Sequence of PD-ECGF from Different Sources

unprocessed PD-ECGF	MAALMTPGTGAPPAPGDFS...
placenta-purified PD-ECGF	TPGTGAPPAPGDFS...
platelet-purified PD-ECGF	APPAPGDFS...
E. coli expressed PD-ECGF	GSPGIPPAPGDFS...

Amino-Terminal Sequence of Recombinant PD-ECGF. T_4 DNA polymerase, used to trim the 3′ overhand, removed five bases from the 5′ terminus of PD-ECGF cDNA after digestion with *Apa*I. Proteolytic digestion of the GST–PD-ECGF fusion protein leaves an extra five amino acids (Figure 1) due to sequences following the thrombin cleavage site (Smith & Johnson, 1988). The combined result is that the recombinant PD-ECGF differs from platelet-purified PD-ECGF at its amino terminus by having Ala replaced, and from placental purified PD-ECGF by having the hexapeptide Thr-Pro-Gly-Thr-Gly-Ala replaced by the pentapeptide Gly-Ser-Pro-Gly-Ile as shown in Table II.

Stimulation of [^{3}H]Methylthymidine Uptake. Figure 4 shows that recombinant PD-ECGF stimulates [^{3}H]methylthymidine uptake by HUVEC's (Figure 4A) and BACE cells (Figure 4B) in vitro. Uptake was also seen with CPAE's (data not shown). Recombinant PD-ECGF shows a statistically significant response at 10 ng/mL (0.2 nM) and a maximal response at 100 ng/mL (2 nM). The GST–PD-ECGF fusion protein also stimulated [^{3}H]methylthymidine uptake but only at a 10-fold higher concentration; i.e., a response was seen at 2 nM and a maximal response at 20 nM (data not shown).

Although thrombin is a mitogen for some cells, it is not a mitogen for endothelial cells (Gospodarowicz et al., 1978), and no response was seen in control experiments that utilized the amount of thrombin that would carry over into the assay from the proteolytic cleavage.

In order to characterize growth stimulation by recombinant PD-ECGF, endothelial cells were incubated with the growth factor for periods of up to 10 days. A number of experiments were performed including assays with microvascular endo-

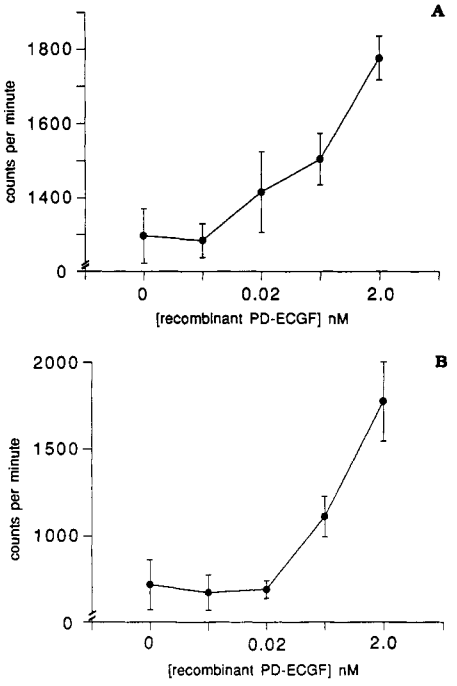

FIGURE 4: Apparent mitogenic activity of recombinant PD-ECGF measured by [^{3}H]methylthymidine uptake. (A) HUVEC's and (B) quiescent BACE cells were incubated for 44 h with recombinant PD-ECGF. [^{3}H]Methylthymidine was then added for a further 4 h prior to cell harvesting and counting (n = 5 ± SEM).

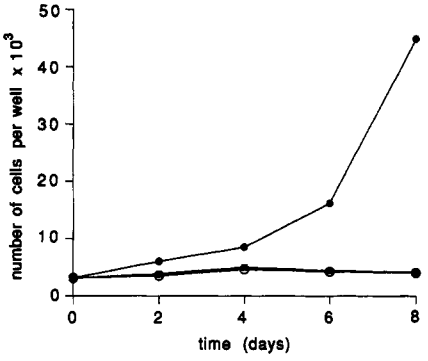

FIGURE 5: Effect of bFGF and PD-ECGF on the growth of BACE cells. Control 10% FCS (open circles), 1 ng/mL bFGF (filled circles), and 100 ng/mL PD-ECGF (filled squares). Quiescent BACE cells in 6-well plates were treated with fresh growth factor containing media on alternate days (n = 2).

thelium (BACE) and large vessel endothelium (HUVEC's and CPAE's). Assays were performed with 0.5%, 2%, and 10% FCS in the presence and the absence of 1 ng/mL basic fibroblast growth factor to examine possible synergy between growth factors. The cells were also allowed to quiesce after seeding and prior to adding the growth factors for 24 h, 7 or 10 days. Relative to controls, no significant change in cell numbers was observed with recombinant PD-ECGF in any experiment. Figure 5 shows data with BACE cells.

Effect of PD-ECGF on Long-Term Uptake of [^{3}H]-Methylthymidine. An observation that led us to investigate whether PD-ECGF was affecting the availability of extracellular thymidine was the [^{3}H]methylthymidine uptake assay shown in Figure 6. Here the experiment was different than that described above, in that both the growth factor and [^{3}H]-methylthymidine were added to the endothelial cells simultaneously and incubated for 48 h before harvesting and counting. The result is opposite to that seen on 4-h pulsing with [^{3}H]methylthymidine in that PD-ECGF appears to inhibit thymidine uptake. Nevertheless, there was no change

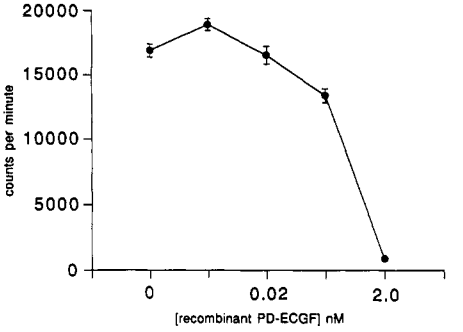

FIGURE 6: Effect of long-term exposure of endothelial cells to recombinant PD-ECGF and [^{3}H]methylthymidine. Ten-day quiescent BACE cells were incubated with recombinant PD-ECGF and [^{3}H]methylthymidine for 48 h. The cells were then harvested and counted as described (n = 5 ± SEM).

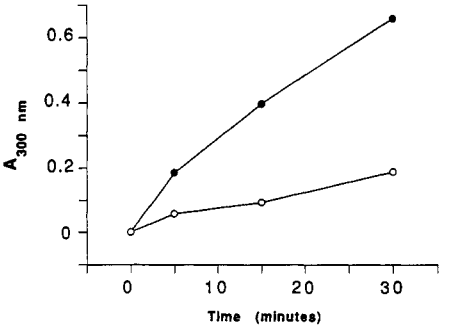

FIGURE 7: Thymidine phosphorylase activity of PD-ECGF at 10 mg/mL (filled circles) and 1 mg/mL (open circles). Release of thymine from thymidine was monitored at 300 nm (Schwartz, 1978).

in total cell number, despite the fact that there was a decrease of over 90% in [^{3}H]methylthymidine uptake by the endothelial cells. The fusion protein was again 10-fold less active than recombinant PD-ECGF (data not shown).

Identification of Thymidine Phosphorylase Activity. The amino acid sequence of the human PD-ECGF is 39% homologous to that of *E. coli* thymidine phosphorylase (Furukawa et al., 1992). That PD-ECGF has thymidine phosphorylase activity was confirmed by monitoring the release of thymine from thymidine at 300 nm. Thymine has a molar extinction coefficient 3.4 × 10^3 times greater than that of thymidine at 300 nm (Schwartz, 1978). Figure 7 shows that recombinant PD-ECGF exhibits thymidine phosphorylase activity, with an activity of 12.8 nmol of thymine released per minute per microgram of protein at 37 °C. This was found to be comparable to that of thymidine phosphorylase from *E. coli* which under the same conditions had an activity of 46 nmol of thymine released per minute per microgram of protein. Purification of thymidine phosphorylase from human placenta has been reported (Yoshimura et al., 1990). The pure protein had a specific activity of 191.4 mmol of thymine released per hour per milligram of protein, which corresponds to 3.2 nmol of thymine released per minute per microgram of protein. This value is similar to that of recombinant PD-ECGF.

Effect of E. coli Thymidine Phosphorylase on [^{3}H]-Methylthymidine Uptake Assay. In order to determine whether the thymidine phosphorylase activity of recombinant PD-ECGF can account for the effect on thymidine uptake by endothelial cells, *E. coli* thymidine phosphorylase was assayed in the same way. BACE cells were incubated with *E. coli* thymidine phosphorylase for 48 h followed by pulsing with [^{3}H]methylthymidine for 3 h (Figure 8A). Alternatively, the cells were incubated with thymidine phosphorylase and [^{3}H]methylthymidine simultaneously (Figure 8B). As with

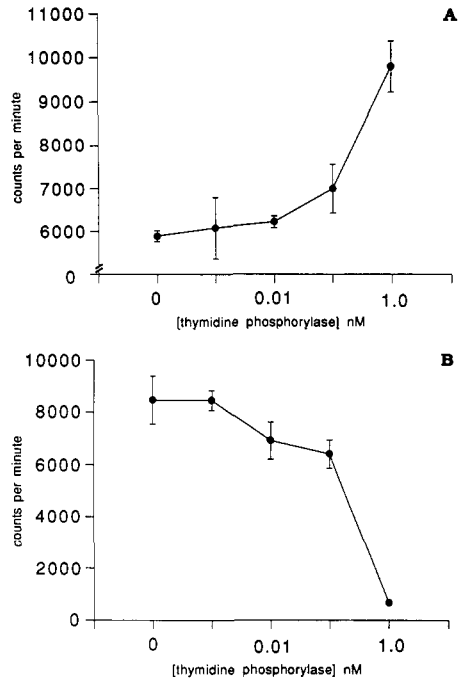

FIGURE 8: Effect of recombinant PD-ECGF on endothelial cell [^{3}H]-methylthymidine uptake can be reproduced with the *E. coli* enzyme thymidine phosphorylase. (A) BACE cells were incubated with thymidine phosphorylase for 44 h followed by addition of [^{3}H]-methylthymidine for 4 h, or (B) BACE cells were incubated with thymidine phosphorylase and [^{3}H]methylthymidine simultaneously for 48 h before harvesting and counting (n = 5 ± SEM).

PD-ECGF, both stimulation and inhibition of thymidine uptake were seen depending on the incubation conditions. *E. coli* thymidine phosphorylase had no effect on cell growth.

DISCUSSION

Expression of eukaroytic proteins in *E. coli* as a fusion protein has the advantages of increasing solubility and stability (Schein, 1991). The GST fusion expression vector has been used for the expression of several eukaryotic proteins (Guan & Dixon, 1991; Kaelin et al., 1991) whereby it allows one-step purification of the expressed protein in high yields. Here, the vector was used for the expression of PD-ECGF, and pure soluble protein was obtained after cleavage at 200 μg/L of culture. The yield is somewhat low if compared to other eukaryotic proteins expressed in *E. coli* (Smith & Johnson, 1988). This is due to reduced solubility and possibly some toxicity. Nevertheless, it is 10 000 times higher than that obtained from blood, and it overcomes the complex multistep purification (Table I). An active form of PD-ECGF has also been expressed in yeast (Finnis et al., 1992); however, purification of yeast recombinant PD-ECGF was not reported.

A general strategy to increase the solubility of an *E. coli* expressed eukaryotic protein is to lower the fermentation temperature, usually to 30 °C (Schien, 1991). We found that induction of exponentially growing bacteria at 15 °C rather than 37 °C yielded substantially more of the soluble protein (Figure 3). It is possible by lowering the temperature that the protein is synthesized more slowly, permitting correct folding, analogous to slow refolding of a peptide by removal by dialysis of a denaturing agent such as guanidine hydrochloride (Marston & Hartley, 1990).

Recombinant PD-ECGF stimulates [^{3}H]methylthymidine uptake by endothelial cells at 0.2 and 2 nM, similar to that purified from platelets (Miyazono et al., 1987). This confirms that the extra amino-terminal amino acids do not alter its activity. The stimulation of thymidine uptake by recombinant PD-ECGF is seen only when the growth factor is preincubated for 24–48 h with cells prior to addition of [^{3}H]methylthymidine. In contrast, when PD-ECGF and [^{3}H]methylthymidine are added simultaneously and cells harvested 24–48 h later. PD-ECGF was found to inhibit uptake of [^{3}H]-methylthymidine in a dose-dependent fashion (see below). There have been many pitfalls reported for [^{3}H]methylthymidine uptake as a measure of cell proliferation (Maurer, 1981; Ho et al., 1990). For this reason, it is essential to confirm results from [^{3}H]methylthymidine uptake assays with other measurements (such as cell counting) before concluding that changes in [^{3}H]methylthymidine uptake reflect effects on cell proliferation. We report that there was no detectable increase in the total cell number in vitro in response to recombinant PD-ECGF, and, indeed, so far there has been no such report in response to PD-ECGF purified from platelets (Miyazono et al., 1987), placenta (Usuki et al., 1990), or that expressed in yeast (Finnis et al., 1992). However, angiogenic responses implicate endothelial cell proliferation in response to PD-ECGF in vivo (Ishikawa et al., 1989).

Figure 7 confirms that PD-ECGF, as suggested by sequence homology, has thymidine phosphorylase activity that is comparable to that of the *E. coli* enzyme. Thymidine phosphorylase catalyzes the phosphorylitic breakdown of thymidine to thymine and deoxyribose 1-phosphate in the presence of orthophosphate (Schwartz, 1978), and this may explain the effects of PD-ECGF on [^{3}H]methylthymidine uptake by cells. Thus, when cells are incubated with PD-ECGF and [^{3}H]methylthymidine simultaneously, the [^{3}H]-methylthymidine is hydrolyzed before it is taken up by the cells, and we see the decrease in apparent uptake (Figure 6). In contrast, when the cells are first incubated with PD-ECGF, thymidine in the culture media is hydrolyzed, and then when the cells are subsequently pulsed with [^{3}H]methylthymidine, those exposed to PD-ECGF and so depleted of thymidine take up more of the label than do controls (Figure 4).

PD-ECGF has been shown to be angiogenic on the chick chorioallantoic membrane (Ishikawa et al., 1989). Further, transfection of PD-ECGF into a1-1 cells (3T3 fibroblasts transfected with the human Ha-ras gene) gave transfectants which formed more highly vascularized tumors in nude mice compared to tumors of the parental a1-1 cells (Ishikawa et al., 1989). This was despite the fact that the PD-ECGF gene had no sequence coding for a secretion peptide, and it is unclear as to how the molecule exits the a1-1 cells. Although the tumors from PD-ECGF transfectants were reported to be more vascular than controls, no data were presented concerning their relative rates of growth. Preliminary experiments have shown that recombinant PD-ECGF does elicit an angiogenic response in the rat sponge model (T.-P. D. Fan, D. E. Hu, A. Moghaddam, and R. Bicknell, unpublished observations). Further studies are in progress.

High-level expression of thymidine phosphorylase in lymphocytes, reticulocytes, and tumors (Zimmerman & Seidenberg, 1964) has prompted speculation that it may play a role in proliferation and/or differentiation of leukocytes as well as in tumor proliferation (Yoshimura et al., 1990). In addition, studies dating back 10 years have shown that thymidine phosphorylase activity is elevated in the plasma of tumor-bearing animals and in the plasma of cancer patients relative to that of healthy subjects (Pauly et al., 1977, 1978).

Our results show that PD-ECGF does not induce angiogenesis by direct stimulation of endothelial proliferation but does so by some, as yet, unidentified indirect mechanism which

could well involve the molecule's thymidine phosphorylase activity. Indeed, nucleoside metabolites have been implicated to play a role in some forms of vascular growth (Morris et al., 1989). The identification of the thymidine phosphorylase activity of PD-ECGF together with bacterial expression and one-step purification will help to elucidate its role in angiogenesis.

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Registry No. Thymidine phosphorylase, 9030-23-3; thymidine, 50-89-5.