

# Epiregulin is a potent vascular smooth muscle cell-derived mitogen induced by angiotensin II, endothelin-1, and thrombin

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**ABSTRACT** Vasoactive GTP-binding protein-coupled receptor agonists such as angiotensin II (AII), endothelin-1 (ET-1), and  $\alpha$ -thrombin ( $\alpha$ -Thr) have been reported to indirectly stimulate vascular smooth muscle cell (VSMC) proliferation by regulating the expression of one or more autocrine growth factors. Using ion-exchange, gel-filtration, and reverse-phase chromatographic purification methods, we isolated a major mitogenic protein present in AII-stimulated rat aortic smooth muscle (RASM) cell conditioned medium. Twenty N-terminal amino acids of the purified peptide were identified, and they had 75% amino acid sequence identity with mouse epiregulin, an epidermal growth factor (EGF)-related growth factor. We cloned the cDNA for rat epiregulin to determine its pattern of expression in G-protein-coupled receptor agonist-stimulated cells and confirm its activity as a mitogen. After treatment of RASM cells with AII, ET-1, or  $\alpha$ -Thr for 1 h, induction of two epiregulin transcripts was observed, including a 4.8-kb transcript and a novel transcript of approximately 1.2 kb. Recombinant rat epiregulin was strongly mitogenic for RASM cells, stimulating DNA synthesis to levels similar to those induced by serum or platelet-derived growth factor and approximately 3-fold above that observed with saturating concentrations of EGF. In addition, epiregulin caused rapid EGF receptor activation in RASM cells. However, relative levels of EGF receptor tyrosine phosphorylation stimulated by epiregulin were less than those induced by EGF or betacellulin. Taken together, these results indicate that epiregulin is a potent VSMC-secreted mitogen, induced in common by AII, ET-1, and  $\alpha$ -Thr, that may contribute to VSMC proliferation and vascular remodeling stimulated by vasoactive agonists.

Vascular smooth muscle cell (VSMC) proliferation is an important component of arterial remodeling in diseases of the vasculature, including atherosclerosis, hypertension, and restenosis after percutaneous transluminal coronary angioplasty (PTCA). Certain GTP-binding protein-coupled receptor (GPCR) agonists [e.g., angiotensin II (AII), endothelin-1 (ET-1), and  $\alpha$ -thrombin ( $\alpha$ -Thr)] have been implicated in the regulation of VSMC proliferation in these pathological conditions. In cultured rat aortic smooth muscle (RASM) cells, these agonists stimulate DNA synthesis (1–7); however, the cellular response is delayed several hours in comparison with cells stimulated with growth factors such as platelet-derived growth factor (PDGF) or basic fibroblast growth factor (FGF) (5–7). This “delayed” response correlates with the accumulation of mitogenic factors in the conditioned medium of the RASM cells. It has therefore been postulated that GPCR agonists convey their mitogenic stimulus through the expres-

sion of endogenous growth factors. In support of this concept, AII, ET-1, and  $\alpha$ -Thr have been reported to stimulate the expression of several growth factors, including PDGF-AA (8–10), basic FGF (11–13), heparin-binding epidermal growth factor (EGF)-like growth factor (14), transforming growth factor  $\beta_1$  (15), and insulin-like growth factor 1 (16). However, little direct evidence has been reported to implicate any of these factors as a major autocrine mediator of VSMC proliferation.

In the present report, we used protein purification and amino acid sequencing methods to identify epiregulin, an EGF-related growth factor, as a major mitogenic protein constituent from AII-stimulated RASM cell conditioned medium. Epiregulin was originally identified as a growth-inhibitory factor from conditioned medium of the murine tumor cell line NIH 3T3/clone T7 (17). It was subsequently demonstrated to act as a mitogen for various cell types, including fibroblasts and hepatocytes. More recent studies have shown that epiregulin expression *in vivo* is very restricted. Besides its constitutive expression in certain tumor cell lines and the developing mouse embryo, significant levels of epiregulin transcripts in human tissues are limited to placenta, macrophages, and to a lesser degree, heart (17, 18). However, these results provide little insight into the normal regulation or physiological function of epiregulin. In this report, we demonstrate that epiregulin acts as a potent VSMC-derived mitogen and that its expression is regulated by GPCR agonists, including AII, ET-1, and  $\alpha$ -Thr. Induction of epiregulin expression in VSMCs therefore may represent one mechanism by which these agonists stimulate cell proliferation in certain vascular diseases, including arteriosclerosis and post-PTCA restenosis.

## MATERIALS AND METHODS

**Cells and Reagents.** RASM cells were cultured as previously described (19). COS-7 (monkey) and Swiss 3T3 (mouse) cells

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: AII, angiotensin II; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; EGF, epidermal growth factor; ET-1, endothelin-1; FGF, fibroblast growth factor; GPCR, GTP-binding protein-coupled receptor; hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase; PDGF, platelet-derived growth factor; PTCA, percutaneous transluminal coronary angioplasty; P-Tyr, phosphotyrosine; RACE, rapid amplification of cDNA ends; RASM, rat aortic smooth muscle;  $\alpha$ -Thr,  $\alpha$ -thrombin; VSMC, vascular smooth muscle cell.

Data deposition: The sequence for rat epiregulin reported in this paper has been deposited in the GenBank database (accession no. AF074952).

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were obtained from the American Type Culture Collection. Recombinant human PDGF-BB and betacellulin were purchased from R & D Systems. Murine EGF was purchased from Collaborative Biomedical Products. AII and ET-1 were obtained from Peninsula Laboratories. Human  $\alpha$ -Thr was from Enzyme Research Laboratories. DNA primers were obtained from Genosys Biotechnologies. SuperScript Preamplification System for First Strand cDNA Synthesis was purchased from GIBCO/BRL. *Taq* and *ExTaq* DNA polymerases were from Boehringer Mannheim and Takara Shuzo, respectively. [*meth*-<sup>3</sup>H]Thymidine (50–90 Ci/mmol; 1 Ci = 37 GBq) and [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol) were obtained from DuPont/NEN.

**Purification of Epiregulin from AII-Stimulated RASM Cell Conditioned Medium.** Five liters of conditioned medium from RASM cells treated for 9 h with AII was prepared as described previously (19). The media was adjusted to 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), filtered, and concentrated 13-fold. Samples were subjected to exhaustive diafiltration (25 mM Tris-HCl, pH 8.1, at 4°C in 0.1% CHAPS) and then applied to an FPLC HR16/10 column (Pharmacia Biotech) containing a Macro Prep High Q anion-exchange support (Bio-Rad) at a flow of 3 ml·min<sup>-1</sup>. Bound proteins were eluted with a linear NaCl gradient and fractions were assayed for activity in a Swiss 3T3 mitogenesis assay as previously described (19). Mitogenic fractions were pooled, concentrated 70-fold, and applied to an equilibrated (25 mM Tris-HCl, pH 7.8, at 25°C in 150 mM NaCl/0.1% CHAPS) HR10/30 Superdex-75 gel-filtration column (Pharmacia Biotech), using a flow rate of 0.5 ml·min<sup>-1</sup>. Selected active fractions from two similar gel-filtration column runs were pooled, concentrated 125-fold, and applied to a narrow-bore C<sub>4</sub> column (Vydac, 2.1 × 150 mm) at a flow of 0.1 ml·min<sup>-1</sup>. Bound proteins were eluted with a 5–65% (vol/vol) acetonitrile gradient in 0.1% trifluoroacetic acid, and fractions (150  $\mu$ l) were neutralized with 2 M Tris-HCl, pH 9. Fractions were purged of volatile organic molecules and applied to a 20% *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) minigel (Promega protocols). Separated proteins were electroblotted to a poly(vinylidene difluoride) (PVDF) membrane, visualized with Coomassie brilliant blue R-250, and excised for N-terminal amino acid sequencing.

**Amino Acid Sequencing of Purified Epiregulin.** PVDF-immobilized proteins were sequenced in a pulsed-liquid protein sequencer (Applied Biosystems, model 476A) using manufacturer-released cycle programs. Phenylthiohydantoin (PTH) amino acid derivatives were separated by reverse-phase HPLC over a 5  $\mu$ m, PTH-C18 column (Applied Biosystems; 2.1 × 220 mm) using a sodium acetate/tetrahydrofuran/acetonitrile gradient for elution. Data were quantitated with chromatogram analysis software (Applied Biosystems, model 610A). Peptide sequences were searched against the GenPept, GeneSeqP, NRL, PIR, and Swiss-Prot databases.

**Cloning of Rat Epiregulin.** Template cDNAs for all PCRs were prepared from oligo(dT)- or dT-adaptor-primed poly(A)<sup>+</sup> RNA isolated from AII-stimulated RASM cells. All PCR products were subcloned into a TA cloning vector (Invitrogen), and sequences of at least three individual clones were confirmed by ABI PRISM Dye Terminator Cycle sequencing (Perkin-Elmer). To obtain the entire coding region for rat epiregulin peptide precursor, a three-step strategy was employed. First, the cDNA for mature rat epiregulin peptide was amplified by reverse transcription-PCR using degenerate primers based on the murine amino acid sequence for epiregulin (20). Since a fragment of the expected size was absent, DNA products from the initial PCR using primers P1 (5'-GGNGARWSNGARGAYAAAYTGYAC-3') and P2 (5'-CKRCARAARTARTADATRCANCC-3') were reamplified in a second PCR using a nested primer P3 (5'-GTNCARATGGARGAYGAYCC-3') together with primer P2 to obtain

mature epiregulin peptide cDNA. Additional sequence information for rat epiregulin mRNA was then obtained by means of the rapid amplification of cDNA ends (RACE) technique (21). The entire coding region for rat epiregulin peptide precursor was amplified by using primers P10 (5'-TATGAATTCGTCCAGCGCTTTGCGAGC-3') and P11 (5'-AGT-TCTAGATTAGACTTGTGGCAACCCTGG-3'), based on sequences generated from RACE. To avoid mutations, we used a single round of PCR to amplify the cDNA for rat epiregulin precursor (94°C, 30 s; 57°C, 30 s; 72°C, 30 s for 10 cycles and 94°C, 30 s; 68°C, 30 s; 72°C, 30 s for 20 cycles). For mammalian expression of epiregulin protein, epiregulin precursor cDNA was inserted between the *EcoRI*-*XbaI* sites of pcDNA3.1(+) (Invitrogen) to obtain the construct pcDNA31.rERpp.

**RNA Extraction and Northern Blot Analyses.** Poly(A)<sup>+</sup> RNA was isolated from quiescent cultures of RASM cells stimulated with AII (1  $\mu$ M), ET-1 (100 nM), or  $\alpha$ -Thr (10 nM) for various times as previously described (19). Relative levels of epiregulin mRNA were quantitated by phosphorimage analysis (Storm 860, Molecular Dynamics) and normalized against a human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) probe.

**Recombinant Rat Epiregulin Protein Expression.** COS-7 cells were electroporated either with pcDNA3.1(+) vector DNA or with pcDNA31.rERpp DNA as described (Bio-Rad protocols). After recovery (48 h), conditioned medium was prepared by incubating cells for 20 h in serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 nM selenium sulfite and antibiotics. Conditioned media samples were collected, filtered, concentrated 100-fold, and assayed for activity in the RASM cell mitogenesis assay (19).

**Immunoprecipitation and Immunoblot Analyses.** Cell lysates obtained from serum-starved RASM cells were immunoprecipitated with an anti-phosphotyrosine (anti-P-Tyr) antibody (4G10, Upstate Biotechnology) and analyzed by immunoblotting as described previously (22). Prior to lysis, cells either were left untreated or were stimulated for 5 min with PDGF-BB (50 ng/ml), EGF (100 ng/ml), betacellulin (100 ng/ml), or 2× concentrated conditioned media samples obtained from transfected COS-7 cells as described above. Duplicate immunoblots were probed separately with anti-P-Tyr or goat polyclonal antibodies directed against the EGF receptor (SC-03G, Santa Cruz Biotechnology). Immunoreactive protein bands were visualized by using <sup>125</sup>I-labeled staphylococcal protein A (Amersham), followed by autoradiography.

## RESULTS

**Purification and Identification of Rat Epiregulin from the Conditioned Medium of AII-Stimulated RASM Cells.** Anion-exchange chromatography (Fig. 1A) served as the initial step in the purification of mitogenic proteins from 5 liters of conditioned medium obtained from AII-stimulated RASM cells. Fractions containing a significant level of mitogenic activity that formed a single broad peak (nos. 8–16, Fig. 1A) were selected, pooled, and concentrated for further purification by gel-filtration chromatography (Fig. 1B). Three major peaks of mitogenic activity were identified by this procedure (A, B, and C, Fig. 1B). Fractions with relatively high specific mitogenic activity (nos. 22–29 of peak C, Fig. 1B and Table 1) were pooled and concentrated for further purification through a reverse-phase column (absorbance profile not shown), resulting in the elution of a single peak of mitogenic activity (fraction nos. 19–23, Fig. 1C). Proteins from each fraction subsequently were resolved by SDS/PAGE and visualized by silver staining (Fig. 1D). A 5-kDa protein band correlating with mitogenic activity (fraction no. 22, Fig. 1D) was submitted for N-terminal amino acid sequencing. Approximately 4.6 pmol of

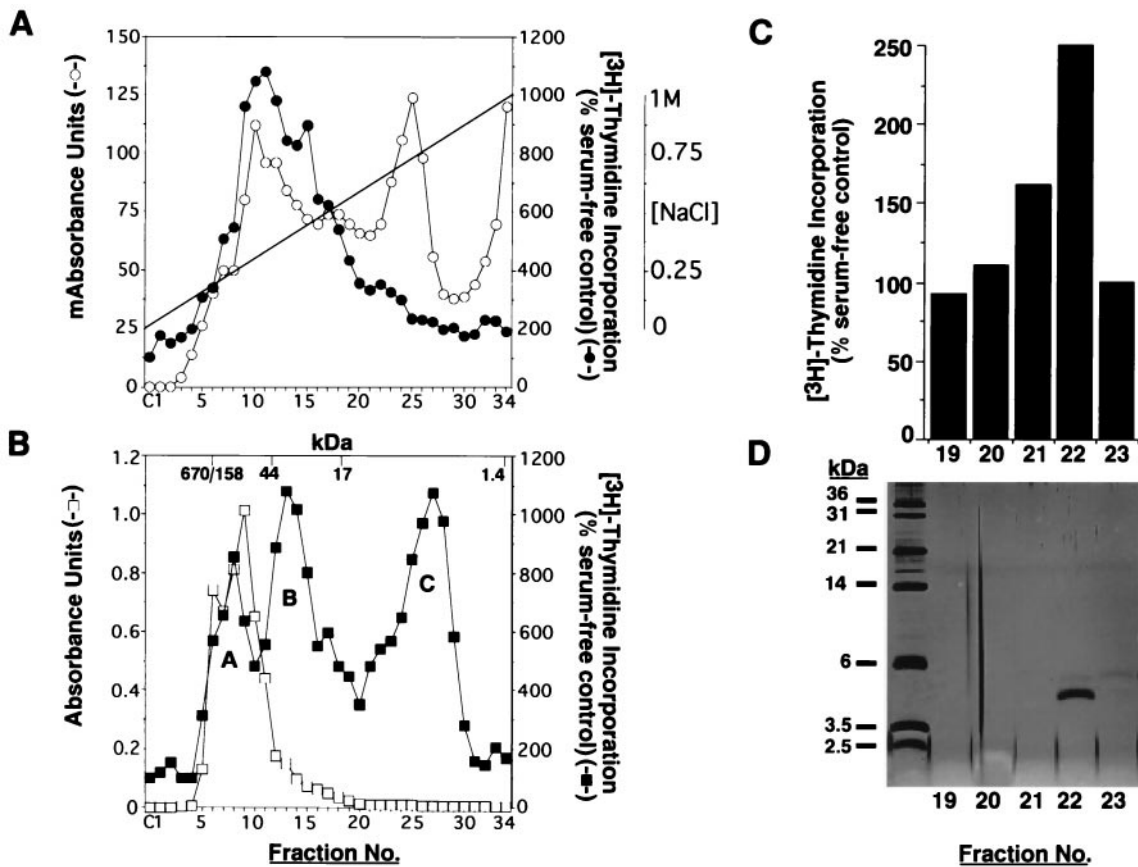


FIG. 1. Purification of rat epiregulin from the conditioned medium of AII-stimulated RASM cells. (A) Five liters of AII-stimulated RASM cell conditioned medium was concentrated and applied to an equilibrated anion-exchange column. Fractions (3 ml), eluted with a NaCl gradient (absorbance at 280 nm, ○), were assayed (50  $\mu$ l/0.5 ml) for mitogenic activity in Swiss 3T3 cells (●). (B) Mitogenic fractions from A (nos. 8–16) were concentrated and applied to a gel exclusion column (absorbance at 280 nm, □) calibrated with protein standards (thyroglobulin and gamma globulin, 670 kDa and 158 kDa, respectively; ovalbumin, 44 kDa; myoglobin, 17 kDa; vitamin B<sub>12</sub>, 1.35 kDa). Fractions (0.35 ml) were assayed for mitogenic activity (25  $\mu$ l/0.5 ml) in Swiss 3T3 cells (■). Mitogenic fractions from two gel exclusion column runs (nos. 22–29, peak C) were concentrated and applied to a C<sub>4</sub> reverse-phase column. Recovered proteins were used for N-terminal amino acid sequencing. In a similar C<sub>4</sub> column run, selected fractions (nos. 19–23) were assayed for mitogenic activity (50  $\mu$ l/0.5 ml) in Swiss 3T3 cells (C). Remaining fractions were resolved by SDS/PAGE and proteins were visualized by silver staining (D).

this protein was detected (Table 1), and the amino acid sequence of the N terminus was determined to be NH<sub>2</sub>-VLITKXSSDMDGYXLHGHI-, X indicating cycles where no phenylthiohydantoin derivative of an amino acid could be detected. Because the sample was not alkylated, these cycles suggest the presence of individual cysteine residues. A computer search using the above amino acid sequence was performed against several protein databases; the search matched 15 of 20 amino acids with murine epiregulin, an EGF-related growth factor (17, 20).

**PCR Amplification and Characterization of a Short Transcript Encoding Rat Epiregulin.** Degenerate oligonucleotide primers, based on the published sequence for murine epiregu-

lin (20), were used to amplify a 267-bp portion of rat epiregulin cDNA (nucleotides 268–534, Fig. 2A) that included the coding sequence for mature epiregulin peptide (nucleotides 301–438, Fig. 2A). The deduced amino acid sequence for mature rat epiregulin peptide (amino acids 56–101, Fig. 2A) contained the N-terminal amino acid sequence that we identified in our purified peptide (underlined, Fig. 2A). 5' and 3' RACE-PCR produced 567 additional base pairs of rat epiregulin cDNA (nucleotides 1–267 and 535–834, respectively, Fig. 2A) that included in-frame start and stop codons (nucleotides 136–138 and 622–624 respectively, Fig. 2A). Eight-hundred and thirty-four nucleotides of rat epiregulin cDNA consensus sequence were assembled (Fig. 2A), including the entire ORF and 5' and

Table 1. Summary of RASM cell-derived mitogen purification

| Purification step     | Protein recovered, $\mu$ g | Total activity, units* | Specific activity, units/ $\mu$ g | Activity recovered, % | Purification factor |
|-----------------------|----------------------------|------------------------|-----------------------------------|-----------------------|---------------------|
| Conditioned medium    | 14,250 <sup>†</sup>        | 2490                   | 0.139                             | 100                   | —                   |
| High Q anion exchange | 2,464 <sup>†</sup>         | 960                    | 0.39                              | 39                    | 3                   |
| Superdex-75           | 2.3 <sup>‡</sup>           | 144                    | 63                                | 5.8                   | 453                 |
| C <sub>4</sub> HPLC   | 0.024 <sup>§</sup>         | 5.7 <sup>¶</sup>       | 238                               | 0.23                  | 1,712               |

\*One unit of activity is defined as the amount of sample required to stimulate [<sup>3</sup>H]thymidine incorporation to a level 50% of that observed for serum in Swiss 3T3 cells.

<sup>†</sup>Determined by protein assay kit (Bio-Rad).

<sup>‡</sup>Estimated by silver staining of SDS/PAGE-resolved protein.

<sup>§</sup>Blotted protein band determined by amino acid composition.

<sup>¶</sup>Activity attenuated by low-pH conditions.



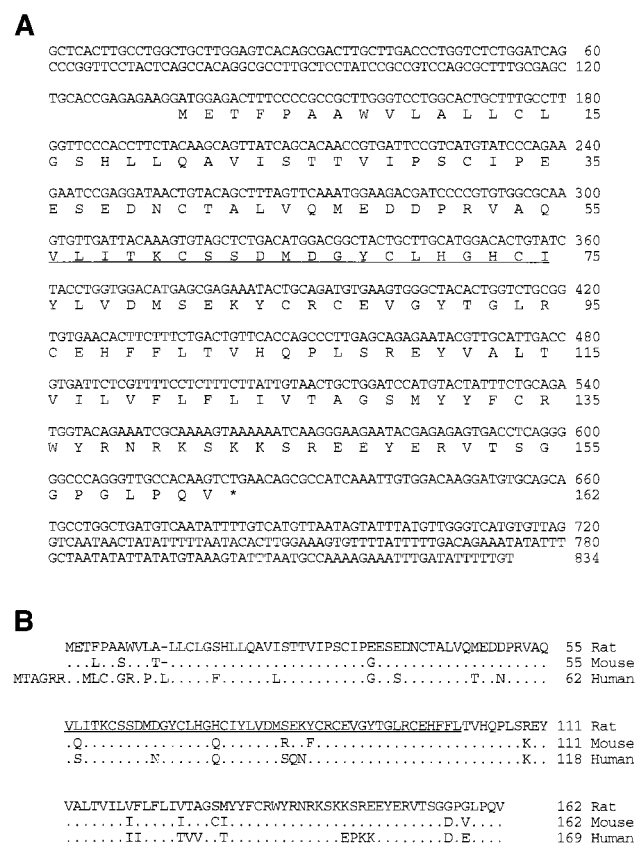


Fig. 2. Nucleotide sequence of a rat epiregulin cDNA transcript and the deduced amino acid sequence of rat epiregulin precursor protein. (A) Nucleotide sequence of rat epiregulin cDNA. The underlined amino acid sequence indicates residues that were identified by N-terminal amino acid sequencing. (B) Alignment of rat, mouse, and human epiregulin polypeptides. The mature rat epiregulin peptide is underlined (amino acids 56–101). A period indicates that an amino acid is identical to the one in the rat peptide at a given position; a hyphen indicates that no corresponding amino acid occupies the indicated position.

3' untranslated sequences. These data indicate the presence of a previously unreported, short epiregulin transcript, discrete from the 4.8-kb murine mRNA (20). Subsequent Northern analyses confirmed the presence of this shorter epiregulin transcript in GPCR-agonist-stimulated RASM cells (see below, Fig. 3A). The predicted amino acid sequence for the mature form of rat epiregulin has 87% and 91% amino acid identity to human and murine epiregulin, respectively (Fig. 2B).

**Induction of Epiregulin in RASM Cells Stimulated with the GPCR Agonists AII, ET-1, and  $\alpha$ -Thr.** After stimulation of quiescent RASM cells with GPCR agonists AII, ET-1, and  $\alpha$ -Thr, we observed a rapid, transient induction of epiregulin mRNA. Levels of the 4.8-kb transcript were induced 5-, 7-, or 9-fold, respectively, above unstimulated levels within 1 h (Fig. 3A and C). Furthermore, AII, ET-1, and  $\alpha$ -Thr also induced a shorter, 1.2-kb, epiregulin transcript after a 1-h stimulation (Fig. 3A). After 3 h, induction of the 4.8-kb transcript increased to 9- and 13-fold above unstimulated levels in cells treated with AII and  $\alpha$ -Thr, respectively. In contrast, epiregulin mRNA was reduced to unstimulated levels in RASM cells treated for either 3 or 6 h with ET-1. Expression of the short transcript diminished after a 3-h treatment with either AII or  $\alpha$ -Thr, and interestingly, resolved as a smaller transcript compared with those observed after 1 h. Levels of both the 4.8- and 1.2-kb transcripts returned to unstimulated levels in RASM cells after a 6-h stimulation with either AII or  $\alpha$ -Thr.

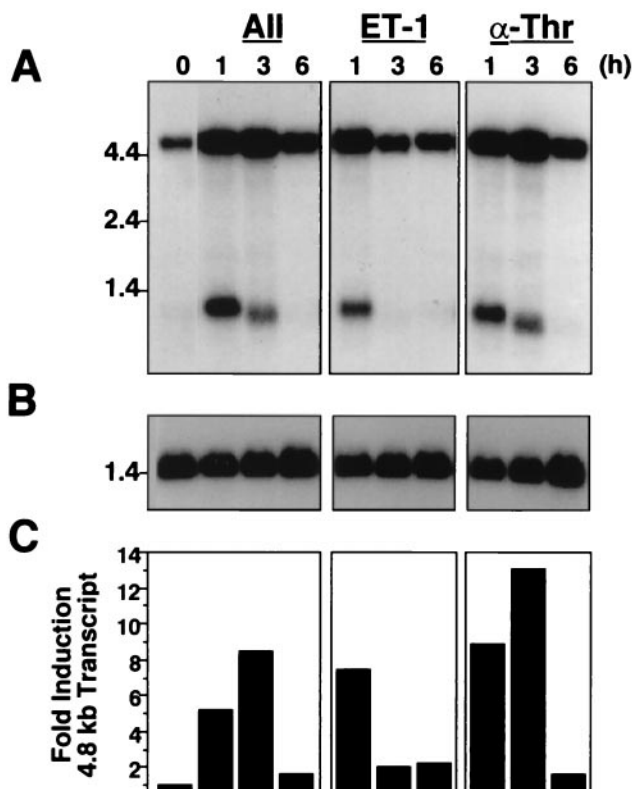


Fig. 3. Induction of epiregulin mRNA by AII, ET-1, and  $\alpha$ -Thr. Poly(A)<sup>+</sup> RNA samples were prepared from serum-starved RASM cells that were either untreated or stimulated with AII (1  $\mu$ M), ET-1 (100 nM), or  $\alpha$ -Thr (10 nM) for the indicated times. (A and B) RNA blots (3  $\mu$ g per lane) were probed with random-primed <sup>32</sup>P-labeled cDNA containing rat epiregulin (A) or hGAPDH (B). Numbers on left are kb. (C) Epiregulin (4.8-kb transcript) expression was quantitated by phosphorimage analysis and normalized by comparison with hGAPDH levels (B). Results are representative of independent experiments performed on two separate occasions.

**Expression and Activity of Recombinant Rat Epiregulin.** To determine whether recombinant rat epiregulin was mitogenic for RASM cells, we transiently transfected a eukaryotic expression vector, pcDNA31.rERpp, containing the coding sequence for rat epiregulin precursor, into COS-7 cells. Conditioned medium obtained from these cells exhibited strong mitogenic activity for RASM cells (Fig. 4, bars B–D) as well as human aortic smooth muscle cells (D.S.T., X.C., and C.J.M., unpublished results). In contrast, conditioned medium obtained from cells transfected with control vector had no significant mitogenic activity (Fig. 4, bar A). In these experiments we observed that the relative level of [<sup>3</sup>H]thymidine incorporation stimulated by 0.5 $\times$  epiregulin conditioned medium (Fig. 4, bar B) was approximately 200% of that induced by EGF and essentially equivalent to the response elicited by serum (10%, vol/vol). Remarkably, 2 $\times$  epiregulin conditioned medium (Fig. 4, bar D) stimulated DNA synthesis to a level comparable to saturating concentrations of PDGF-BB in the RASM cells, and approximately 150% of that stimulated by serum.

**Induction of EGF Receptor Tyrosine Phosphorylation by Recombinant Epiregulin.** Experiments were performed to evaluate the ability of recombinant epiregulin to stimulate EGF receptor activation in cultured RASM cells. In these studies, conditioned media samples obtained from COS-7 cells transfected with full-length rat epiregulin were used as a source of recombinant protein. The ability of epiregulin to stimulate EGF tyrosine phosphorylation was compared with the EGF receptor agonists EGF and betacellulin (23). PDGF-BB ho-

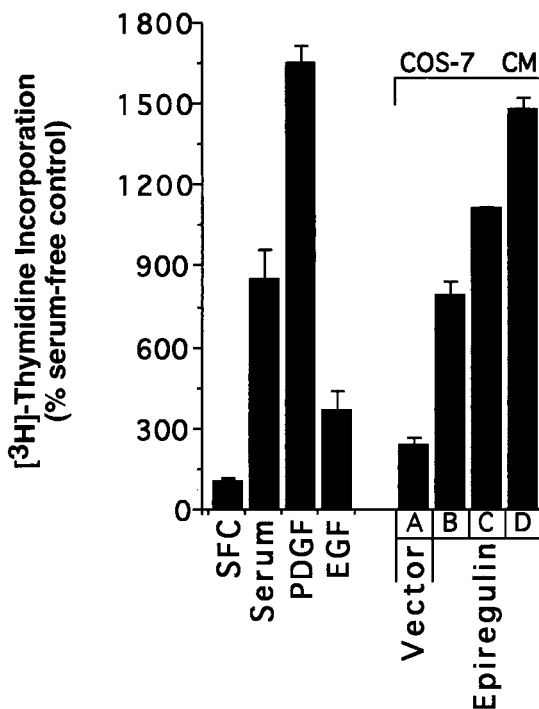


FIG. 4. Mitogenic activity of recombinant rat epiregulin on RASM cells. Quiescent RASM cells were either left untreated (SFC, serum-free control) or stimulated for 24 h with PDGF-BB (50 ng/ml), EGF (50 ng/ml), serum (10%, vol/vol), control COS-7 cell conditioned medium (bar A, 2× final concentration) or conditioned medium from epiregulin-transfected COS-7 (bars B, C, and D, 0.5×, 1×, and 2× final concentration, respectively). Results are expressed as a percentage [3H]thymidine incorporation in comparison to serum-free control levels. Values shown are the mean ± SEM from experiments performed two times with triplicate samples in each experiment.

modimers and conditioned medium from COS-7 cells transfected with an empty expression vector were included as controls. With the exception of control COS-7 conditioned media samples, addition of each of the agonists led to increased protein tyrosine phosphorylation in quiescent RASM cells (Fig. 5A). PDGF-BB induction of PDGF receptor (185-kDa) tyrosine phosphorylation was especially pronounced. In contrast, EGF, betacellulin, and epiregulin each stimulated tyrosine phosphorylation of similar 170-kDa protein bands, which were confirmed to be the EGF receptor by anti-EGF receptor immunoblotting (Fig. 5B). Of note, we consistently observed that epiregulin stimulated EGF receptor tyrosine phosphorylation less effectively than either EGF or betacellulin.

**DISCUSSION**

In previous studies we demonstrated that physiological concentrations of vasoactive GPCR agonists stimulated delayed mitogenic responses in rat VSMCs that temporally correlated with the presence of increased mitogenic activity in cellular conditioned media samples (5, 6, 19). Although we observed the expression of several growth factors in stimulated VSMC cells (e.g., PDGF-AA, transforming growth factor β<sub>1</sub>, activin A), rigorous functional experiments excluded these and many other known factors as significant mitogenic components in conditioned media. Accordingly, our goal was to purify and characterize important mitogens secreted by GPCR agonist-stimulated VSMCs.

In this report, epiregulin was identified as a major mitogenic constituent of AII-stimulated RASM cell conditioned medium. Thus, the presence of highly purified epiregulin corre-

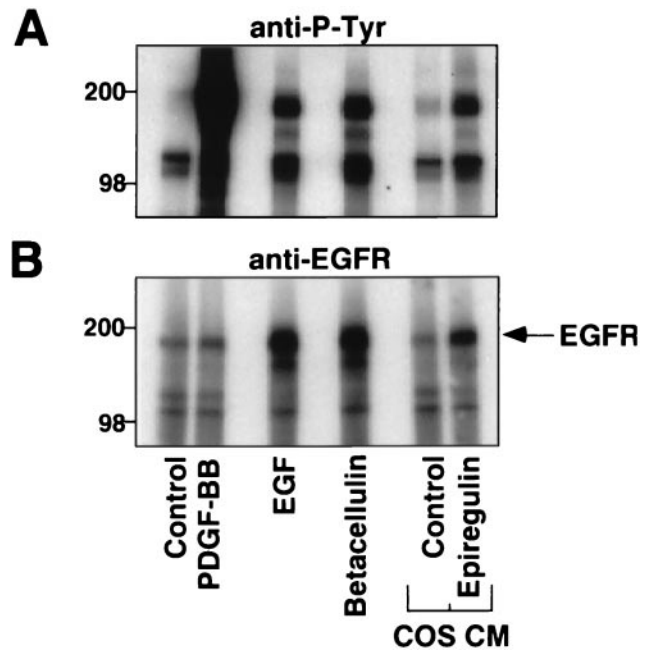


FIG. 5. Recombinant rat epiregulin stimulates EGF receptor activation in cultured RASM cells. Serum-starved RASM cells were either untreated (Control) or stimulated for 5 min with PDGF-BB (50 ng/ml), EGF (100 ng/ml), betacellulin (100 ng/ml), or 2× concentrated conditioned media (CM) obtained from epiregulin or mock-transfected COS-7 cells as indicated. Cell lysates were immunoprecipitated with anti-P-Tyr antibodies, and recovered proteins were resolved by SDS/PAGE. Duplicate gels were immunoblotted with either anti-P-Tyr (A) or anti-EGF receptor (B) antibodies. Anti-P-Tyr-recovered EGF receptors are indicated by the arrow (B). Data are representative of independent experiments performed on two separate occasions.

lated with recovered mitogenic activity after several chromatographic steps, including reverse-phase chromatography. On the basis of the protein purification results (Table 1), effective mitogenic concentrations of epiregulin for either Swiss 3T3 or RASM cells were determined to be between 0.3 and 0.6 ng/ml, a range comparable to that of other growth factors, including basic FGF and PDGF (24, 25). To confirm that epiregulin was the factor responsible for the purified mitogenic activity, the rat cDNA for epiregulin was cloned and expressed in COS-7 cells. Recombinant epiregulin was a strong VSMC mitogen with activity greater than that of serum (10%, vol/vol) and nearly equivalent to PDGF-BB. These data indicate that epiregulin alone is capable of stimulating DNA synthesis to levels previously observed in RASM cells stimulated with AII, ET-1, or α-Thr (5–7). Furthermore, the induction of epiregulin mRNA transcripts in response to the GPCR agonists closely correlated with their relative mitogenic activities on RASM cells (5–7). Thus, mRNA and thymidine incorporation levels in RASM cells were highest after α-Thr stimulation, followed by AII and ET-1. Taken together, these data support epiregulin as a common GPCR-agonist-induced autocrine mitogen for RASM cells that may be an important mediator of VSMC proliferation *in vivo*.

In response to AII, ET-1, and α-Thr, two discrete epiregulin mRNA transcripts were induced in RASM cells. Only the 4.8-kb transcript had been detected previously in surveys of various normal and tumor cell lines and tissues (18, 20). The exact mechanism by which the shorter 1.2-kb transcript arose is unclear. One possibility is that the novel transcript could be a result of alternative polyadenylation. In this regard, a search of the 3' untranslated region of the mRNA failed to show any canonical polyadenylation sites; however, we cannot exclude the possibility of an atypical polyadenylation signal. It should

be noted that multiple potential polyadenylation signals exist in both the mouse and human 4.8-kb transcripts. In addition, a number of mRNA destabilizing signals [e.g., AUUUA, UUAUUUA(U/A)(U/A)] are present in both the mouse and human transcript (18, 20). Thus it is possible that generation of shorter transcripts may help to eliminate these destabilizing signals, leading to more efficient translation. However, we cannot rule out alternative splicing as a source for the shorter transcript because the genomic DNA sequence of epiregulin is not yet available.

Recent reports have implicated transactivation of EGF receptors in the rapid signaling pathways stimulated by certain GPCRs (26, 27). Interestingly, both EGF receptor and ErbB-4 have been identified as molecules that specifically bind epiregulin (28, 29). In this regard, Shelly *et al.* (30) demonstrated that epiregulin had widespread selectivity for the ErbB family of receptor tyrosine kinases, as it differentially activated every ErbB receptor combination except for homodimers of ErbB-2 and ErbB-3. These authors also noted that epiregulin's affinity for ErbB homo-/heterodimer combinations was markedly lower than other ErbB ligands even though its biological responses were greater. This difference was attributed to a reduced rate of receptor recycling after epiregulin stimulation that allowed for prolonged intracellular signaling (30, 31).

In the present study, we confirmed that recombinant epiregulin stimulated EGF receptor activation in RASM cells (Fig. 5). Furthermore, consistent with the studies cited above, we observed that epiregulin was less effective than EGF or betacellulin in the induction of EGF receptor tyrosine phosphorylation despite the fact that it was a stronger mitogen (Fig. 4). Thus, relative levels of EGF receptor tyrosine phosphorylation did not correlate with the biological effects of epiregulin. In related studies (D.S.T., X.C., and C.J.M., unpublished results), we were unable to detect ErbB-4 expression in RASM cells by either protein immunoblotting or Northern analysis, suggesting that this receptor is not required for the mitogenic effects of epiregulin in these cells. These data suggest that EGF receptors, either as homodimers or in heterodimeric complexes with ErbB-2, ErbB-3, or other unidentified molecules, serve as the principal mitogenic receptors for epiregulin in VSMCs.

Although epiregulin's exact function *in vivo* is not known, it displays a highly restricted expression pattern suggesting that it may be an important mediator of localized cell proliferation (17, 18). In this regard, constitutive expression of epiregulin in some malignant cells supports a role for this protein in tumorigenesis, possibly by directly stimulating cell proliferation and/or angiogenesis associated with tumor expansion. The discovery of physiologically regulated epiregulin expression in VSMCs strengthens the potential connection between this molecule and vascular pathologies. For example, vasoactive GPCR agonists have been implicated in post-PTCA restenosis, supporting epiregulin as a secondary effector molecule in arterial neointimal formation. Furthermore, since epiregulin transcripts have been identified in human macrophages and the heart (18), this strong vascular mitogen may contribute to chronic fibroproliferative vascular diseases, including atherosclerosis. It is also possible that GPCR-regulated epiregulin expression may be identified in other cell types with the appropriate capacity to respond to these stimuli. Therefore, additional studies of epiregulin expression *in vivo* are required to clarify the role of this potent mitogen in both normal physiology and disease.

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