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A Novel Immediate-Early Response Gene of Endothelium Is Induced by Cytokines and Encodes a Secreted Protein

LAWRENCE B. HOLZMAN,[1] RORY M. MARKS,[2,3] AND VISHVA M. DIXIT[3]*

Department of Internal Medicine, Division of Nephrology,[1] Division of Rheumatology,[2] and Department of Pathology,[3] The University of Michigan Medical School, Ann Arbor, Michigan 48109

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We have previously described the cloning of a group of novel cellular immediate-early response genes whose expression in human umbilical vein endothelial cells is induced by tumor necrosis factor α in the presence of cycloheximide. These genes are likely to participate in mediating the response of the vascular endothelium to proinflammatory cytokines. In this study, we further characterized one of these novel gene products named B61. Sequence analysis of cDNA clones encoding B61 revealed that its protein product has no significant homology to previously described proteins. Southern analysis suggested that B61 is an evolutionarily conserved single-copy gene. B61 is primarily a hydrophilic molecule but contains both a hydrophobic N-terminal and a hydrophobic C-terminal region. The N-terminal region is typical of a signal peptide, which is consistent with the secreted nature of the protein. The mature form of the predicted protein consists of 187 amino acid residues and has a molecular weight of 22,000. Immunoprecipitation of metabolically labeled human umbilical vein endothelial cell preparations revealed that B61 is a 25-kilodalton secreted protein which is markedly induced by tumor necrosis factor.

The vascular endothelium plays an active central role in the process of acute inflammation (reviewed in reference 31). Under the influence of proinflammatory cytokines such as tumor necrosis factor (TNF) α and interleukin-1β (IL-1), endothelial cells convert from an anticoagulant to a procoagulant phenotype (2, 3, 32, 38), induce leukocyte adhesion and chemotaxis (4, 37), and secrete cytokines important for hematopoiesis (7, 39, 40), leukocyte activation, and smooth muscle cell proliferation (28). Although TNF and IL-1 are not directly chemotactic, they induce endothelial cells to secrete chemotactic factors such as interleukin-8 (monocyte-derived neutrophil chemotactic factor) (43) and monocyte chemoattractant protein 1 (13, 35, 48, 49). Adhesion of leukocytes to stimulated endothelium is facilitated by the cytokine-mediated increased plasma membrane expression of intercellular adhesion molecule 1 (15) and endothelial leukocyte adhesion molecule 1 (5). The activated endothelium further contributes to establishing the inflammatory response by secreting additional IL-1 (29) as well as interleukin-6 (41) and several colony-stimulating factors (7, 39, 40), which induce leukocyte activation and participate in the differentiation and proliferation of T and B cells.

To better understand the complex response of endothelial cells to proinflammatory cytokines, we have used differential hybridization to clone a group of eight immediate-early response genes, whose expression in cultured human umbilical vein endothelial cells (HUVE) is induced by TNF-α in the presence of cycloheximide (CHX) (13). By limiting the analysis to immediate-early response genes, we hoped to identify gene products that could play pivotal roles in mediating endothelial response to cytokines. DNA sequencing permitted the identification of four of these gene products. Two chemotactic cytokines, interleukin-8 and monocyte chemoattractant protein 1, as well as two adhesion molecules, endothelial leukocyte adhesion molecule 1 and intercellular adhesion molecule 1, were found to be primary response gene products of TNF-stimulated endothelial cells (13, and unpublished data). The cloning of these important mediators of inflammation and the discovery that they are primary response genes affirmed the validity of the approach taken.

Our analysis of TNF-induced, endothelial cell-derived primary response genes also led to the cloning of four novel genes. We report here on the further characterization of one of these novel gene products, B61.

As previously described (13), the 1.55-kilobase B61 mRNA is induced within 15 min in HUVE after TNF stimulation. This induction is largely a result of increased transcriptional rate as assessed by nuclear run-on experiments. Steady-state message expression peaks at 2 h and is superinduced in the presence of CHX. B61 message is similarly induced by IL-1 and lipopolysaccharide but not by gamma interferon or by endothelial cell growth factor. Preliminary data (unpublished) reveals that B61 expression is not cell type specific; rather, B61 mRNA expression is induced by TNF and IL-1β in both keratinocytes and IMR-90 fibroblasts in culture.

In this report, we describe the full-length cloning and sequencing of B61. B61 is a novel protein without significant homology to other known proteins. Moreover, we found that B61 is the secreted protein product of TNF-stimulated endothelial cells.

MATERIALS AND METHODS

Reagents. Recombinant human TNF-α purified from *Escherichia coli* was a gift from Genentech (S. San Francisco, Calif.). Synthetic oligonucleotides were produced on a DNA-101 oligonucleotide synthesizer (Biotix, Danbury, Conn.) or Applied Biosystems 380A oligonucleotide synthesizer (Foster City, Calif.).

Endothelial cells. HUVE were used for all studies. They were isolated from human umbilical cords by the method of Jaffe (22) and cultured as described previously (14). Cells were identified as endothelial on the basis of their morphology at confluence, positive immunofluorescence with anti-

* Corresponding author.

body to human von Willebrand factor (Calbiochem), and positive staining with fluorescienated *Ulex europaeus* I lectin (Vector Laboratories, Inc., Burlingame, Calif.).

Endothelial cells for RNA extraction and metabolic radiolabeling were grown to confluence in either 150-cm^2 flasks (Corning Glass Works, Corning, N.Y.) or 75-cm^2 dishes (Falcon, Cockeysville, Md.). Cells were used between passages 2 and 4. The night before extraction or labeling, culture medium was removed, cells were washed twice with Hanks buffered salt solution containing calcium and magnesium (GIBCO), and culture medium lacking endothelial cell growth factor and heparin was added. TNF (20 ng/ml) was added as described in individual experiments and incubated for the indicated periods. CHX, when used, was added at 10 μg/ml 30 min before the addition of TNF (14).

cDNA library screening. Poly(A)$^+$ mRNA isolated from HUVE treated for 4 h with TNF and CHX was used to construct a λgt11 cDNA library as previously described (13). cDNA fragments derived from the 5′ end of B61.0 cDNA were radiolabeled by a random hexanucleotide primer method (16) and used to rescreen this library (13). Hybridization was performed in 50% formamide–5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–3× Denhardt solution (36)–0.25% sodium dodecyl sulfate (SDS)–1 μg of poly(A) per ml–200 μg of salmon sperm DNA per ml–2 × 10^6 cpm of ^{32}P-labeled cDNA per ml at 42°C for 16 h. Filters were washed once at room temperature, followed by two 30-min washes at 65°C in 2× SSC–0.2% SDS. Hybridizing cDNA inserts were isolated and subcloned into pGEM7zf(+) (Promega Biotec, Madison, Wis.) for sequencing.

The polymerase chain reaction was used as described by Jansen et al. (23) as an alternate approach to cloning the 5′ end of the cDNA. Synthetic oligonucleotides corresponding to the region flanking the cloning site in the λgt11 bacteriophage and a region corresponding to the 5′ end of partial B61 cDNA were used as primers (0.2 μM each) to amplify directly from the λgt11 cDNA library with a Cetus Gene Amp Kit. A Perkin-Elmer Thermal Cycler was cycled for 1 min 15 s at 94°C, 1 min at 55°C, and 4 min at 72°C for 35 cycles and followed by a 10-min final extension at 72°C. The amplified material was ligated to PGEM7zf(+) for sequencing.

Primer extension. High-pressure liquid chromatography-purified oligonucleotide primers were 5′ end labeled (specific activity, 5 × 10^6 cpm/pmol) with [γ-^{32}P]ATP and T4 polynucleotide kinase (Boehringer Mannheim), and the labeled product was purified on Nensorb (Dupont) columns. Total RNA (30 μg) derived from TNF- and CHX-treated HUVE was annealed to 1 pmol of labeled primer in a 5 mM phosphate buffer (pH 6.75) containing 5 mM EDTA. The mixture was heated to 90°C, NaCl was added to final concentration of 10 mM, and the mixture was cooled slowly to 30°C. Extension with avian myeloblastosis virus reverse transcriptase (Seikagaku, Rockville, Md.) was done at 43°C for 75 min in the presence of 10 mM dithiothreitol, 60 U of RNasin, 50 mM Tris hydrochloride (pH 8.3), 5.5 mM $MgCl_2$, 10 mM NaCl, and 1 mM deoxynucleoside triphosphates. Labeled products were phenol-chloroform extracted, separated on a denaturing 6% acrylamide sequencing gel, and visualized by autoradiography.

DNA sequencing and analysis. Plasmid DNA prepared by polyethylene glycol precipitation (1) was sequenced by the dideoxy-chain termination method (44) with modified T7 DNA polymerase (Sequenase; U.S. Biochemicals) and synthetic oligonucleotide primers. DNA sequence was assembled and analyzed by the Sequence Analysis Software Package of the Genetics Computer Group (version 6.2) on a VAX computer. Wordsearch and FastA routines with the algorithms of Wilbur and Lipman (47) and Pearson and Lipman (33), respectively, were used in searches of homology to DNA and protein sequences contained within the following databases: GenBank (release 63.0), NBRF/PIR (release 23), DNA Database of Japan (15 May 1990). In addition, the GenBank on-line service was used to search the Swiss Protein Database and translated GenBank (new sequences) on 17 May 1990 (33). Search for internal repeat and analysis of hydropathy were performed by standard methods (6).

Southern blot analysis. Genomic Southern blot analysis was done in a standard fashion (36). Human genomic DNA was isolated from 11B squamous carcinoma cells as described previously (36). Genomic DNAs from rabbit, macaque, rat, mouse, and drosophila were gifts from Paul Killen and Patrick Venta.

Fusion protein construction and expression. Fusion protein vectors containing the *Pst*I-*Hin*dIII fragment of B61 fused in frame to bacterial *trpE* were expressed in *E. coli*, with the use of a pATH-22 vector (12, 42) (see Fig. 6A and Results). Prior to expression, prospective clones were analyzed by restriction digestion to ensure that the cloning site was appropriately re-created and that the reading frame was preserved. Transformed cells were grown in 10 ml of M9 medium containing 1% Casamino Acids, 20 μg of tryptophan per ml, and 150 μg of ampicillin per ml. For induction, cells were pelleted, tryptophan was removed by washing the pellet in tryptophan-free medium, and the culture was resuspended in 100 ml of tryptophan-free medium. After an additional 1 h of incubation, β-indoleacrylic acid was added to 20 μg/ml and the incubation was continued for an additional 4 h. The relatively insoluble fusion protein product was partially purified from bacterial protein by lysing the cell pellet in lysozyme (3 mg/ml), sonicating (three times for 15 s each on ice), and successively washing with 20 ml of ice-cold 0.5% Nonidet P-40 in 0.3 M NaCl, 1 M NaCl in 10 mM Tris hydrochloride (pH 7.5), and 10 mM Tris (pH 7.5). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue R-100 staining.

Preparation of polyclonal immune serum. TrpE-B61 fusion protein was isolated by SDS-PAGE, and the protein bands were lightly stained in water-based Coomassie blue. The appropriate band (approximately 400 μg) was cut out, homogenized, and mixed 1:1 with either complete or incomplete Freund adjuvant. This preparation was injected intramuscularly into the proximal lower extremities of New Zealand White female rabbits. A total of six boost immunizations were given at 4-week intervals. Rabbits were bled 2 weeks after immunizations.

Immune serum was analyzed by immunoblotting as described previously (21). Briefly, appropriate antigen was separated by SDS-PAGE, transferred to nitrocellulose with an LKB Multiphor II semidry electrophoresis apparatus (Pharmacia) according to the instructions of the manufacturer, and blocked overnight in Tris-buffered saline (pH 7.5) containing 3% nonfat dry milk. After incubation in immune serum, blots were probed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) diluted 1:1,000. Blots were developed with color development reagent containing 4-chloro-1-naphthol (Bio-Rad) and dilute hydrogen peroxide according to the directions of the manufacturer.

Metabolic radiolabeling and immunoprecipitation. HUVE were metabolically labeled as described previously (34)

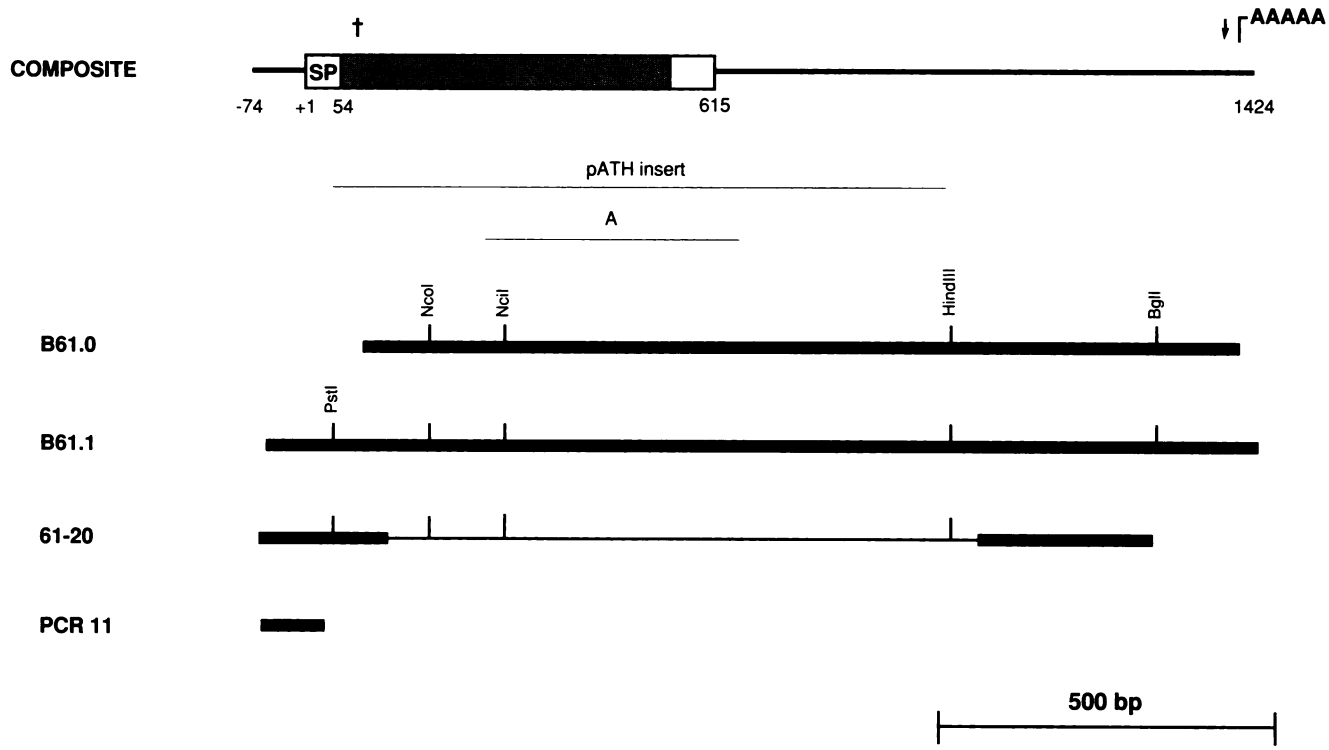

FIG. 1. Schematic representation of B61 mRNA and cloning strategy. A composite B61 mRNA is represented colinear with the four cDNA clones used to derive the B61 sequence. The composite B61 mRNA is depicted on top by the thin line with the predicted coding region expanded. Numbers represent nucleotide bases, with adenosine in the initiation ATG designated +1. Open boxes represent hydrophobic domains, and the stippled central box shows a hydrophilic domain; SP designates a signal peptide. The potential site of N-linked glycosylation is marked above with a dagger (†). The start of poly(A) tail is marked AAAAA, and the arrow preceding this indicates the site of the consensus polyadenylation signal (AAUAAA). The portions of cDNA clones used for genomic Southern blot analyses and as an insert for fusion protein construction are shown above the restriction map of B61 cDNA clones. Bold lines indicate regions of each cDNA clone that were sequenced in both directions. The composite B61 mRNA sequence was derived by sequencing at least two coordinate cDNAs. The only differences in nucleotide sequence lie within the 3′ untranslated region, where nucleotides in B61.0 differed from those in corresponding positions in B61.1 (position 915, A to T; position 1142, A to G). These may represent polymorphisms or errors made by reverse transcriptase during cDNA synthesis. bp, Base pairs.

except that labeling was performed in methionine-free, cysteine-free minimal essential medium (Select-Amine; GIBCO) supplemented with 50 μg of bovine serum albumin per ml and 100 μCi each of [^{35}S]methionine and [^{35}S]cysteine (Amersham) per ml. Conditioned medium was collected and centrifuged to remove cell debris, and protease inhibitors were added to final concentrations of 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 20 μg of pepstatin A per ml. The cell layer was solubilized in 0.5% SDS, and protease inhibitor cocktail was added. Lysate DNA was sheared through a 23-gauge needle. The solubilized cell layer was made 0.1% SDS, 1% Triton X-100, and 0.5% sodium deoxycholate and immunoprecipitated as previously described (34).

For the iodination of HUVE, plates were washed once in cold phosphate-buffered saline and cells were removed from plates by incubation in phosphate-buffered saline containing 1 mM EDTA at 4°C for 15 min. Cells were pelleted at 1,000 × *g* for 5 min and washed three times in 5 ml of cold phosphate-buffered saline. Iodination with 130 μCi of sodium iodide-125 (specific activity, ~17 Ci/mg of I; ICN) was done in an iodogen tube (18) for 1 h on ice. Cells were washed three times in 12 ml of ice-cold 10 mM sodium thiosulfate in phosphate-buffered saline and were finally solubilized in 0.5% SDS and protease inhibitor cocktail was added.

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL, GenBank, and DNA Database of Japan nucleotide sequence databases and has been assigned the accession number M37476.

RESULTS

Molecular cloning and sequencing of B61. DNA probes obtained from the original B61 cDNA, B61.0 (13), were used to rescreen a λgt11 library constructed with poly(A)$^+$ mRNA from HUVE treated with both TNF-α and CHX. Several additional clones were isolated, at least one of which, B61.1, contained the entire open reading frame for B61 (Fig. 1). To clone the 5′ end of the cDNA, we used the polymerase chain reaction with primers containing sequences from the 5′ end of the partial B61 cDNA and sequences flanking the cloning site in the λgt11 vector. The λgt11 cDNA library was used as template DNA. Again, several clones were obtained which allowed confirmation of the 5′-most cDNA sequence. An analytical primer extension (Fig. 2) was done to assess the 5′ extent of the B61 mRNA. As shown, the B61 mRNA extends an additional 25 to 29 bases 5′ to the cloned B61 cDNA.

Structural features. The B61 cDNA sequence and translation of the predicted open reading frame are presented in Fig. 3A. Comparison of these sequences with both protein

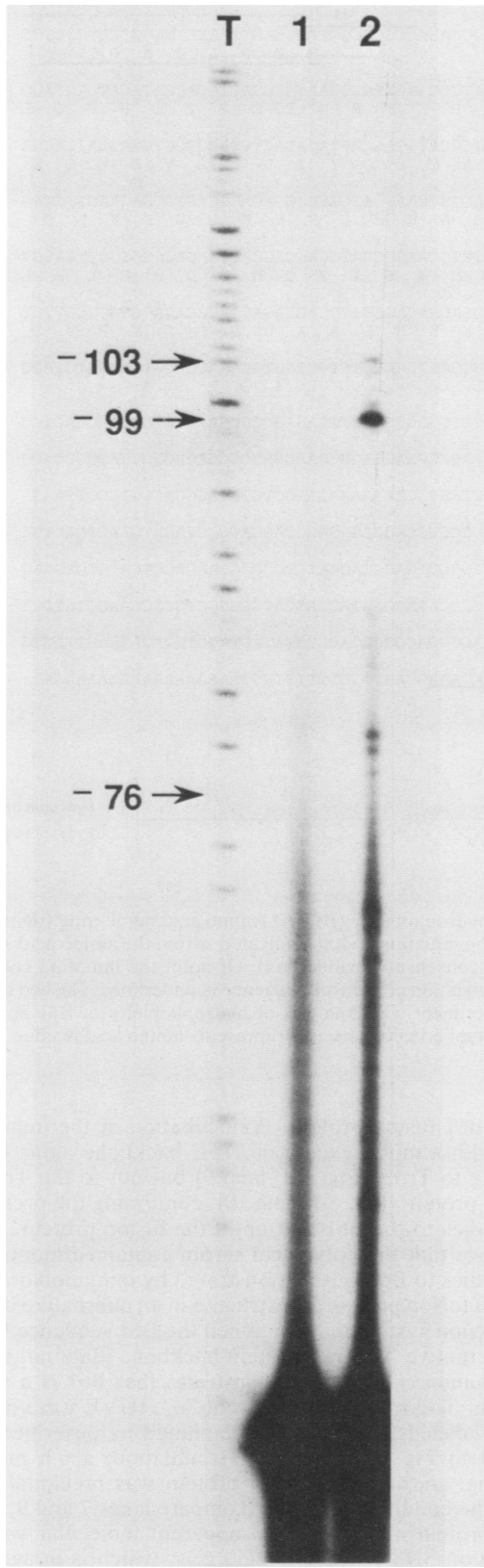

FIG. 2. Primer extension analysis. Synthetic oligonucleotides (see Fig. 3, dashed arrows) were 5′ end labeled and hybridized to 30 μg of total RNA obtained from HUVE pretreated for 2 h with TNF (20 ng/ml) and CHX (10 μg/ml). After reverse transcription, products were resolved on a 6% denaturing acrylamide gel. Shown are nucleotide base markers (lane T), a control experiment with 30 μg of tRNA as the template (lane 1), and primer extension with primer I as described above (lane 2). The primer extension was repeated with a second primer (designated primer II; see Fig. 3) and confirmed the results obtained with the first primer (data not shown). Numbers represent distance in bases from the initiation codon.

and DNA sequence databases failed to identify related gene products of significant homology.

The B61 mRNA (including the uncloned extreme 5′ untranslated region as defined by primer extension) extends over 1,524 nucleotide bases. It includes a 99-base G+C-rich (70% G+C content) 5′ untranslated region, a continuous open reading frame of 615 bases, and a 3′ untranslated region of 810 bases. Within the 3′ untranslated region, a polyadenylation signal is found upstream of the poly(A) tail. No destabilization consensus sequence (UUAUUUAU) is present (9).

The assigned initiation codon represents the 5′-most AUG encountered and is located within a sequence context favorable to translation initiation as defined by Kozak (25). Confirmation of the reading frame determined by the putative initiation AUG is provided by the observation (see below) that a polyclonal immune serum, generated to a fusion protein constructed in the same reading frame, immunoprecipitated specifically a protein of the appropriate molecular weight from human endothelium.

The predicted open reading frame encodes a polypeptide of 205 amino acids with an estimated molecular weight of 24,000. An analysis of hydrophobicity (Fig. 3B) demonstrated that B61 contains markedly hydrophobic N-terminal and C-terminal regions. The 18-amino-acid N-terminal hydrophobic region closely fits von Heinje's consensus for a signal peptide (46). Comparison with known signal peptides reveals that cleavage likely follows the (−3, −1) rule (45) and precedes the aspartic acid residue in position 19. Cleavage of the signal peptide would therefore form a mature protein of 187 amino acids and predicted molecular weight of 22,000.

The C-terminal hydrophobic region spans 23 amino acid residues but does not appear to be a transmembrane domain. Unlike known transmembrane segments, this hydrophobic region is interrupted by several polar amino acids, extends to the extreme C terminus, and is not followed by a cluster of basic amino acid residues. There is structural similarity to the C-terminal region of glycosyl-phospatidylinositol (GPI)-linked membrane-anchored proteins (17).

The remainder of the molecule is predominantly hydrophilic. A single consensus sequence for N-linked glycosylation exists near the N terminus of the mature protein. There appear to be no significant internal repeat motifs as judged by self-comparison by dot-matrix analysis (30).

The B61 gene is unique and conserved. Genomic Southern blots of DNA from 11B human squamous carcinoma cells were hybridized with cDNA probe A or B61.1 (as defined in Fig. 1). The A probe, comprising a 300-nucleotide segment representing the 3′ half of the open reading frame, recognized discrete genomic fragments in each of four genomic DNA restriction digests (Fig. 4). Labeled B61.1 cDNA was used to probe an identical Southern blot (data not shown). Several additional hybridizing fragments were identified. Screening at reduced stringency (2× SSC, room temperature) did not result in the appearance of additional bands. Thus, B61 is a single-copy gene which does not appear to be a member of a family of evolutionarily related proteins.

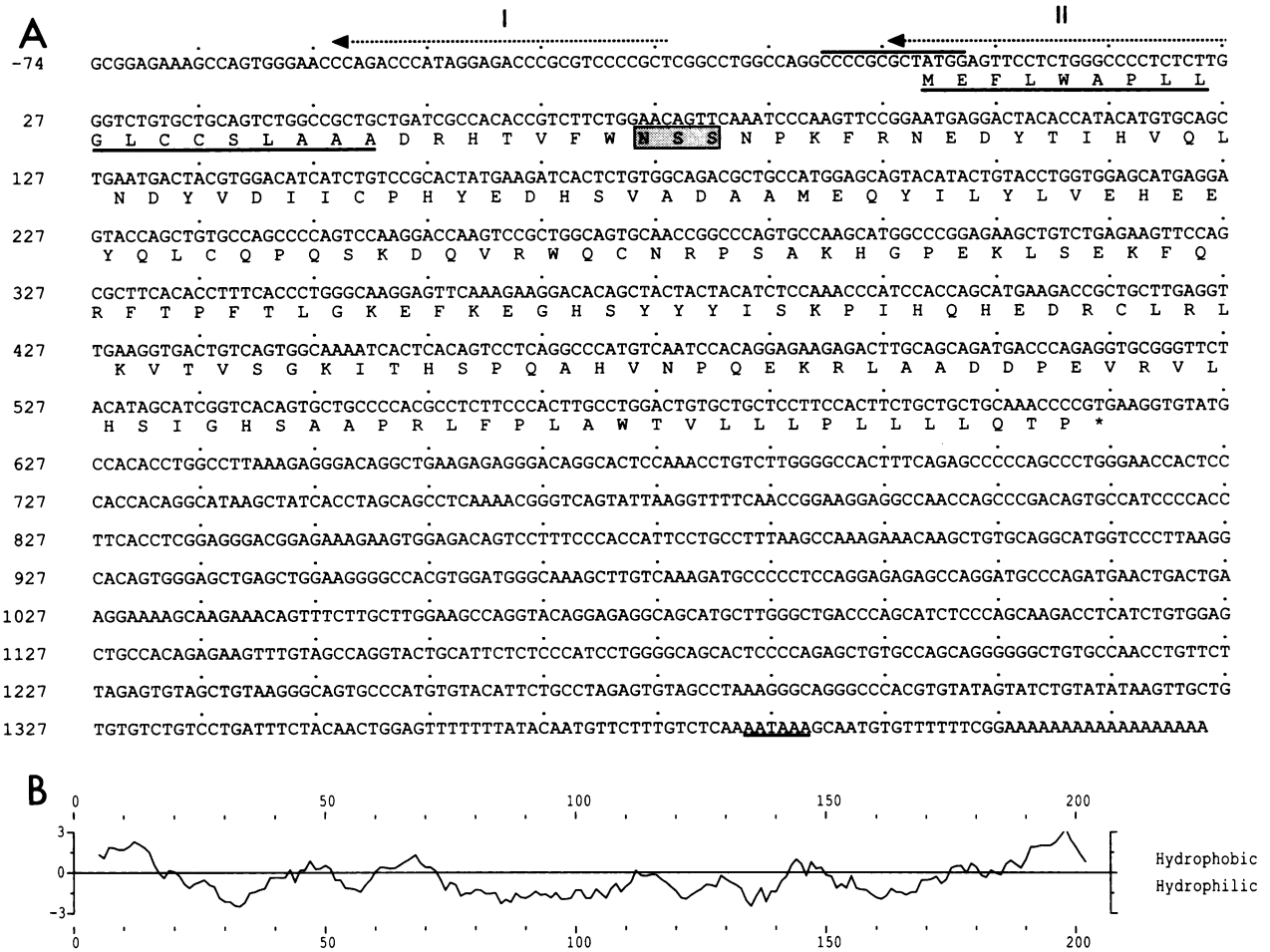

FIG. 3. B61 nucleotide and predicted amino acid sequences (A) and plot of hydrophobicity (B). (A) Amino acid numbering (right margin) begins at the initiator methionine; nucleotide numbering (left margin) begins at the initiation codon. Indicated within the amino acid sequence is the putative signal peptide (underlined) and a single N-linked glycosylation consensus (shaded box). Of note, the initiation codon is in agreement with Kozak's consensus sequence (25) (solid overlined). The polyadenylation consensus sequence is underlined. Dashed overlying arrows (I and II) denote regions used as primers in the primer extension experiment. (B) Analysis of hydrophobicity of B61 amino acid sequence. The plot was generated by the algorithm of Kyte and Doolittle (26). Numbering on abscissa represents amino acid residue. Positive values represent hydrophobic regions. Window of averaging = 9.

Figure 5 shows a Southern blot of *Eco*RI-digested genomic DNA obtained from several animal species. When probed with B61.0, homologous genomic DNA fragments were identified in all species except drosophila. Thus, the sequence and gene structure of B61 appear to have been conserved during evolution.

Generation of polyclonal immune serum to B61. To provide a reagent for further characterization of the B61 protein, we prepared rabbit antiserum to a bacterial fusion protein. A *Pst*I-*Hin*dIII restriction fragment was ligated into a bacterial *trpE* expression vector, fusing the bacterial TrpE protein with the open reading frame encoding amino acid residues 15 to 205 of B61. This construct encoded the entire polypeptide sequence of the predicted mature form of B61. After expression in *E. coli* (Fig. 6A), the insoluble TrpE-B61 fusion protein was dissolved in SDS sample buffer and fractionated by SDS-PAGE and the band was cut from the preparative gels and used to immunize rabbits.

The presence of monospecific antibodies to B61 was determined by immunoblotting (Fig. 6B). The polyclonal immune serum recognized a band corresponding to the TrpE bacterial backbone as well as a band corresponding to the TrpE-B61 fusion protein. Preincubation of the immune serum with a molar excess of TrpE backbone alone blocked binding to TrpE (Fig. 6B, lane 9) but not to the TrpE-B61 fusion protein (Fig. 6B, lane 10), confirming the presence of antibodies to the B61 portion of the fusion protein. Further evidence that the polyclonal serum contained monospecific antibodies to B61 was demonstrated by immunoblotting to a second fusion protein, constructed in an alternative bacterial expression system that contained the B61 sequence fused to an alternative bacterial protein backbone (data not shown).

Immunoprecipitation demonstrates that B61 is a secreted protein. To localize the B61 protein, HUVE were metabolically labeled and immunoprecipitated as described in the legend to Fig. 7. After TNF stimulation, a 6-h metabolic labeling, and a 6-h chase, a protein was precipitated only from the conditioned media (compare lanes 7 and 9, Fig. 7). This protein migrated at an apparent molecular weight of 25,000 on SDS-polyacrylamide gels, which is in agreement with the molecular weight of B61 predicted from primary structure. Precipitation of this band was specifically blocked by preincubating B61 antisera with a molar excess of the TrpE-B61 fusion protein (Fig. 7, lane 8).

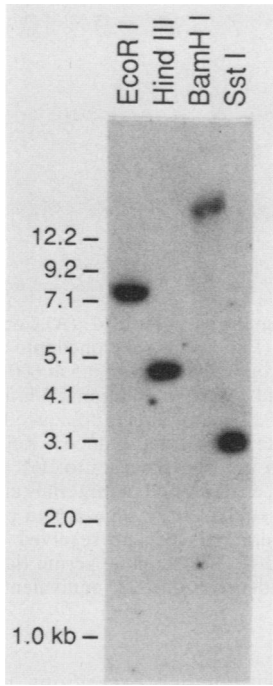

FIG. 4. Genomic Southern analysis demonstrating that B61 is a single-copy gene. Genomic DNA isolated from 11B human squamous carcinoma cells was digested with *Eco*RI, *Hin*dIII, *Bam*HI, or *Sst*I. A 10-μg portion of each sample was resolved on a 0.6% agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled 300-base-pair fragment (Fig. 1, fragment A) under high-stringency conditions (hybridization buffer including 5× SSC and 50% formamide at 42°C; wash buffer including 0.1× SSC and 0.1% SDS at 65°C). Film exposure was for 18 h at −80°C with an intensifying screen.

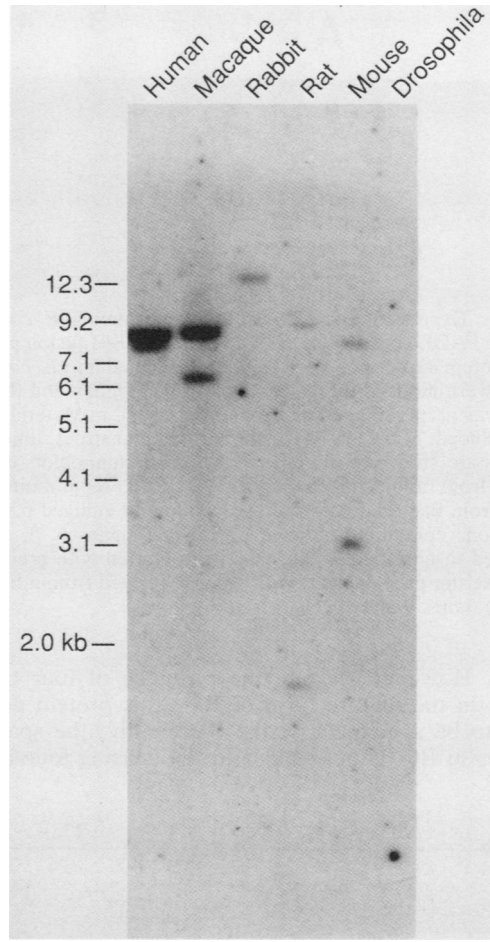

FIG. 5. Genomic Southern analysis demonstrating evolutionary conservation of B61. Genomic DNA (10 μg) isolated from cells representing the various animal species shown was digested with *Eco*RI, resolved on a 0.6% agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled B61.0 cDNA. After hybridization at 42°C in 5× SSC–50% formamide, a successive series of washes from low to high stringency (2× SSC at room temperature to 0.1× SSC at 65°C) were done. Shown is the autoradiograph of the blot after washing at 1× SSC at 55°C. Film exposure was for 3 days at −80°C with intensifying screens. No additional bands were detected on lower-stringency washes in any species including drosophila.

In the pulse-chase experiment presented in Fig. 7, B61 did not appear to be significantly associated with the cell layer. Indeed, a 3-week exposure of this gel failed to allow detection of cell-associated B61. However, given the similarity of the C-terminal hydrophobic region of B61 to the C-terminal regions found in GPI-linked membrane-anchored proteins (17), we utilized an alternative experimental approach to confirm that B61 is not plasma membrane associated. Intact TNF-stimulated HUVE were surfaced iodinated and detergent solubilized, and the solubilized fraction was immunoprecipitated with B61 antiserum. Immunoprecipitated proteins were reduced and resolved by SDS-PAGE and visualized by autoradiography. Labeled B61 was not detected in the immunoprecipitated material. From the accumulated evidence, it appears that B61 is a secreted protein without detectable membrane association.

Finally, B61 expression and secretion is markedly induced after TNF stimulation in HUVE (compare lanes 2 and 7, Fig. 7). This is in concordance with previously published (13) Northern (RNA) blot analysis data which demonstrate significant induction of B61 mRNA after TNF stimulation.

DISCUSSION

B61 is a novel secreted protein whose expression is rapidly induced in endothelial cells after stimulation with the proinflammatory cytokines TNF-α and IL-1β. Comparison of the sequence of the cDNA clones encoding B61 with sequence library data demonstrated that this protein has no significant homology to previously identified proteins. However, the B61 protein has several important structural features. Amino acid sequence analysis revealed that the mature form is a relatively small protein with a predicted molecular weight of 22,000. On reducing SDS-PAGE, B61 migrates as a 25-kDa protein. This difference could be due to glycosylation or other posttranslational modification. Indeed, a single site for N-linked glycosylation exists at the N terminus of the predicted mature form of the protein. As yet, no experimental evidence is available to confirm that B61 is glycosylated at this site.

Members of the small inducible and secreted protein (SIS) superfamily such as human monocyte chemoattractant protein 1 (the human homolog of mouse JE), human platelet factor 4, and human interleukin-8 are all expressed in HUVE in response to activation stimuli and can act as proinflammatory cytokines (27). Like the members of this superfamily, B61 is also a small, secreted, cytokine-inducible gene

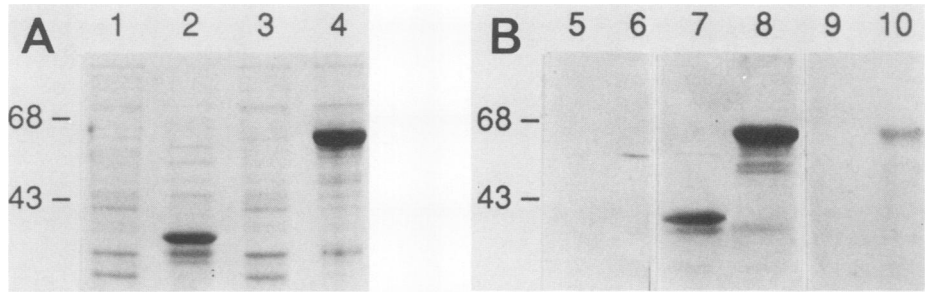

FIG. 6. Expression of pATH-B61 fusion protein in *E. coli* and generation of polyclonal immune serum to B61. (A) Coomassie blue-stained SDS-10% PAGE showing expression of pATH-B61 fusion protein. A B61 *Pst*I-*Hin*dIII insert (Fig. 1) was subcloned into a pATH-22 bacterial fusion protein expression vector and transformed into *E. coli* DH5α cells. After growth to an optical density of 0.5 at 600 nm, the appropriate cultures were induced by the removal of tryptophan and the addition of β-indole acrylic acid. After an additional 4-h incubation, insoluble protein was prepared and analyzed as described in Materials and Methods. Lanes: 1, pATH-22 vector alone, uninduced; 2, pATH22 vector alone, induced; 3, pATH-B61 fusion protein construct, uninduced; 4, pATH-B61 fusion protein construct, induced. Relative molecular size standards are shown to the left ($\times 10^3$). (B) Immunoblots demonstrating specificity of rabbit polyclonal serum to B61. Immune serum was prepared from rabbits injected with gel-purified preparations of the pATH-B61 fusion protein, as described in Materials and Methods. Protein derived from bacterial preparations containing induced pATH-22 alone (lanes 5, 7, and 9; equivalent to lane 2) and protein derived from preparations containing induced pATH-B61 (lanes 6, 8, and 10; equivalent to lane 4) were reduced and resolved on SDS-PAGE and transferred to nitrocellulose. These were blotted with preimmune rabbit serum (lanes 5 and 6), B61 immune serum (lane 7 and 8), or B61 immune serum preincubated with protein derived from a bacterial preparation containing induced pATH-22 (equivalent to eightfold excess of lane 2) (lanes 9 and 10).

product. However, despite the presence of four cysteine residues in the mature form of B61, this protein does not appear to be a member of the SIS family; the spacing of cysteines in B61 is not similar to the spacing found in SIS proteins which with minor exceptions is invariably conserved (8).

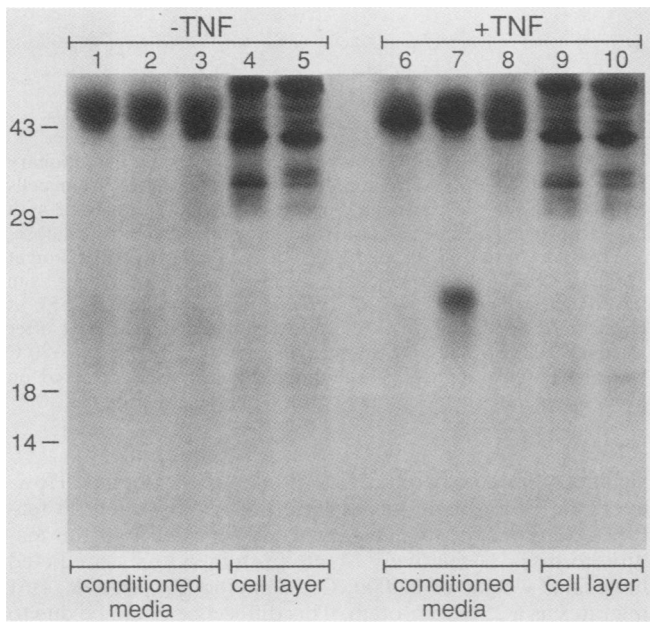

FIG. 7. B61 is a secreted protein. HUVE were deprived of endothelial cell growth factor overnight and then stimulated (lanes 6 to 10) with TNF-α (20 ng/ml) for 2 h. Cells were metabolically labeled with [^{35}S]methionine (100 μCi/ml) and [^{35}S]cysteine (100 μCi/ml) in the presence or absence of TNF-α for 6 h and then chased for 6 h. Conditioned medium (7×10^6 cpm) and cell layers (5×10^7 cpm) were immunoprecipitated as indicated below. The precipitated material was reduced and resolved by SDS-12.5% PAGE and visualized by autoradiography. Lanes: 1, 4, 6, and 9, immunoprecipitated with preimmune rabbit serum; 2, 5, 7, and 10, B61 immune serum; 3 and 8, B61 immune serum preincubated with pATH-B61 fusion protein (equivalent to Fig 6, lane 4). Relative molecular size standards are indicated at left ($\times 10^3$).

Analysis of hydrophobicity showed that B61 is primarily hydrophilic but possesses both an N-terminal and a C-terminal hydrophobic domain. The N-terminal domain has a well-defined signal peptide structure (46) which is consistent with the secreted nature of B61.

The role of the C-terminal hydrophobic domain is unclear. While this segment spans 23 amino acid residues, it appears unlikely that it represents a transmembrane segment since it is interrupted by polar residues. Moreover, no basic amino acid residues typical of a membrane retention signal are found on the C-terminal side of this region. Although no clear consensus sequence exists for GPI-linked membrane-anchored proteins (17), the B61 C terminus bears striking structural similarity to the hydrophobic C terminus of many of these proteins. In GPI-linked proteins, the hydrophobic C terminus is thought to function as a signal for GPI attachment and is cleaved during posttranslational linkage steps (17). Despite this similarity, we have been unable to detect B61 associated with the plasma membrane of TNF-stimulated HUVE in culture. Since GPI-linked proteins such as neural cell adhesion molecule and TAG-1 are found in both a membrane-associated form and a released form (19, 20), it remains possible that B61 also exists in both a plasma membrane-bound and a secreted form under conditions other than those tested. Further, since B61 expression is not endothelial cell specific, a membrane-associated form may be expressed in cells other than HUVE.

TNF dramatically affects endothelial cell function by promoting a pleiotropic response which includes a proinflammatory phenotype. In our investigation of the changes induced by TNF, we limited our analysis to the cloning of cellular immediate-early genes. Such genes undergo rapid and profound induction independent of intermediary protein synthesis. On the basis of the results of earlier work, in which JE and KC (10) as well as c-*myc* and c-*fos* (11, 24) were found to be immediate-early response genes, we postulated that other gene products would be identified which were either paracrine factors essential for mediating the interactions of the activated endothelial cell with its environ-

ment or nuclear regulatory proteins capable of initiating programs within the cell. This hypothesis was borne out by the cloning of the paracrine factors monocyte chemoattractant protein 1 and interleukin-8, as well as two adhesion molecules, ELAM 1 and ICAM 1 (13). All are essential for leukocyte recruitment and activation in acute inflammation. The new secreted protein, B61, may have an equally important role in the complex proinflammatory environment.

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