Ku80 as a Novel Receptor for Thymosin β4 That Mediates Its Intracellular Activity Different from G-actin Sequestering*

Received for publication, September 10, 2007, and in revised form, October 30, 2007 Published, JBC Papers in Press, November 4, 2007, DOI 10.1074/jbc.M707539200

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Our data demonstrate that increased intracellular expression of thymosin β4 (Tβ4) is necessary and sufficient to induce plasminogen activator inhibitor type 1 (PAI-1) gene expression in endothelial cells. To describe the mechanism of this effect, we produced Tβ4 mutants with impaired functional motifs and tested their intracellular location and activity. Cytoplasmic distributions of Tβ4(AcSDKPT/4A), Tβ4(KLKKTET/7A) and Tβ4(K16A) mutants fused with green fluorescent protein did not differ significantly from those of wild-type Tβ4. Overexpression of Tβ4, Tβ4(AcSDKPT/4A) and Tβ4(K16A) affected intracellular formation of actin filaments. As expected, Tβ4(K16A) uptake by nuclei was impaired. On the other hand, overexpression of Tβ4(KLKKTET/7A) resulted in developing the actin filament network typical of adhering cells, indicating that the mutant lacked the actin binding site. The mechanism by which intracellular Tβ4 induced the PAI-1 gene did not depend upon the N-terminal tetrapeptide AcSDKP and depended only partially on its ability to bind G-actin or enter the nucleus. Both Tβ4 and Tβ4(AcSDKPT/4A) induced the PAI-1 gene to the same extent, whereas mutants Tβ4(KLKKTET/7A) and Tβ4(K16A) retained about 60% of the original activity. By proteomic analysis, the Ku80 subunit of ATP-dependent DNA helicase II was found to be associated with Tβ4. Ku80 and Tβ4 consistently co-immunoprecipitated in a complex from endothelial cells. Co-transfection of endothelial cells with the Ku80 deletion mutants and Tβ4 showed that the C-terminal arm domain of Ku80 is directly involved in this interaction. Furthermore, down-regulation of Ku80 by specific short interference RNA resulted in dramatic reduction in PAI-1 expression at the level of both mRNA and protein synthesis. These data suggest that Ku80 functions as a novel receptor for Tβ4 and mediates its intracellular activity.

Thymosin β4 (Tβ4) is the most abundant member of the highly conserved family of acidic polypeptides called β-thymosins. Although it is a typical intracellular polypeptide, it plays numerous roles, both intracellularly and extracellularly. Numerous observations indicate that Tβ4 is involved in adhesion and spreading of fibroblasts (1, 2), differentiation of endothelial cells (3, 4), directional migration of endothelial cells and keratinocytes (5–8), angiogenesis (3–6, 9, 10), wound healing (6, 7, 11), hair follicle growth (8), and apoptosis (12, 13), and has been described to possess anti-inflammatory properties (11, 14). In addition, elevated Tβ4 expression has been observed in various malignant cell lines and tumors (15–17) and its levels seem to be associated with increased tumorigenicity and metastatic potential (10, 13, 18–20). The increased expression of Tβ4 correlates with the invasive capability of the cells, the degree of morphologic transformation, and disintegration of actin filaments (21). Recent studies have also correlated increased Tβ4 expression with potentiated cell growth (13, 22) but this observation seems not to be universal (2, 10).

Tβ4 is considered to be a major actin sequestering molecule, which specifically binds monomeric actin (G-actin) forming a 1:1 complex, or by additionally including profilin, forming a ternary complex (23). Thus, the mechanism by which Tβ4 influences cell proliferation, migration, and differentiation is generally believed to be linked with maintaining a dynamic equilibrium between G-actin and F-actin, critical for the rapid reorganization of the cytoskeleton. Under some conditions Tβ4 can be found in large amounts in extracellular fluids and shows a broad range of biological activities. For example, the Tβ4 level in blood may amount to micromolar concentrations, particularly in prethrombotic states characterized by the enhanced reactivity of blood platelets (24).

Recent observations indicate that Tβ4 can express its activity toward different cells via receptor-mediated mechanisms. Thus, Tβ4 externally added to endothelial cells: (a) induces expression and release of plasminogen activator inhibitor type 1 (PAI-1) by the mechanism involving activation of the mitogen-activated protein kinase cascade leading to enhanced c-Fos/c-Jun binding to the AP-1-like element present in the PAI-1 promoter (25, 26); (b) binds to PINCH and integrin-linked kinase, resulting in activation of the survival kinase Akt, and thus promotes myocardial and endothelial cell migration in the embryonic heart (27) and (c) promotes skin and corneal

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* This work was supported by Grants PBZ-MIN-005/P04/2002/9 and N30101231/0230 from the Polish Ministry of Science and Education. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2. The abbreviations used are: Tβ4, thymosin β4; PAI-1, plasminogen activator inhibitor type 1; siRNA, short interference RNA; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; RT, reverse transcriptase; PBS, phosphate-buffered saline; MS, mass spectrometry; TRITC, tetramethylrhodamine isothiocyanate; GFP, green fluorescent protein.
wound healing through its effects on cell migration, angiogenesis, and possibly cell survival (4, 6, 11).

However, the precise molecular mechanism(s) through which it functions remains unknown. It is not clear whether Tβ4 effects are mediated by (a) extracellular or (b) intracellular receptors, or if (c) Tβ4 is taken up by cells and its activity is manifested after interaction with G-actin and modulation of the actin filament structure. Therefore, in our present studies we attempt to describe intracellular mechanisms by which Tβ4 may activate endothelial cells to up-regulate PAI-1 expression. Particularly, we searched for proteins that may interact in endothelial cells with Tβ4 and thus mediate its activating effects. Based on our affinity binding experiments, co-immunoprecipitation, and proteomic analyses we identified the Ku80 subunit of ATP-dependent DNA helicase II to specifically interact with Tβ4. Specific down-regulation of its expression by short interference RNA (siRNA) abolished the stimulatory activity of Tβ4 toward PAI-1 expression suggesting its contribution in controlling PAI-1 gene activity.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All standard tissue culture reagents including Dulbecco’s modified Eagle’s medium, fetal bovine serum, and Lipofectamine 2000 reagent were from Invitrogen (Eggenstein, Germany). Wizard Miniprep and Maxiprep kits for isolation of plasmid DNA were purchased from Promega Corp. (Madison, WI). Protein A/G-agarose, Enhanced Chemiluminescence (ECL) Western blotting substrate, and BCA Protein Assay Kit were obtained from Pierce. Anti-PAI-1 rabbit polyclonal antibody was from American Diagnostica (Pfungstadt, Germany). Horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody were obtained from Pierce. Anti-PAI-1 rabbit polyclonal antibody and horseradish peroxidase-conjugated goat anti-mouse antibody was purchased from Jackson ImmunoResearch (West Grove, PA). pCMV-Myc and pCMV-Tag were from Clontech. Anti-Ku80 rabbit polyclonal antibody and horseradish peroxidase-conjugated goat anti-mouse antibody was from American Diagnostica (Pfungstadt, Germany). pEGFP-N1-Tβ4(AaSDKP/F4A) and pEGFP-N1-Tβ4(K16A) were used for total RNA, extracted by the TRIzol reagent method (Invitrogen). 

**Cell Cultures**—Human endothelial cell line EA.hy 926, derived by fusion of human umbilical vein endothelial cells with continuous human lung carcinoma cell line A549 (28) was obtained as a gift from Professor Cora-Jean S. Edgell (Pathology Department, University of North Carolina, Chapel Hill, NC). This endothelial hybrid cell line is presently the best characterized macrovascular EC line (for review, see Ref. 29). The cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose, supplemented with 10% fetal bovine serum, HAT (100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine), and antibiotics in a 90–95% humidified atmosphere of 5% CO2 at 37 °C.

**Construction of Tβ4 and Its Mutants Fused with EGFP—DNA coding Tβ4 sequence (135 nucleotides) was synthesized (Bionovo, Ulm, Germany) and amplified by PCR using primers 5′-CTCCG-CCTGGGATCATGTGACACACCC-3′ and 5′-CTCAAT-AAGCTTTTACGATTCGCTGC-3′. Then, it was first sub-cloned into BamHI and HindIII sites in pRSETa, amplified from pRSETa-Tβ4 using primers 5′-CTCCCCCTGGAAGCTTATGT-CTGACAAAAC-3′ and 5′-CTCAATGGATCCTTACGATT-CGCCTGC-3′, and subcloned into pEGFP-N1. The resulting pEGFP-N1-Tβ4 was propagated in Escherichia coli, purified with Wizard Midiprep (Promega), and sequenced to confirm the open reading frame. Selected regions of Tβ4 were modified by scanning mutagenesis replacing the chosen amino acid residues by Ala in pRSETa-Tβ4. The following mutants were obtained: (a) Tβ4(AaSDKP/F4A) in which the N-terminal AcSDKP sequence is substituted by alanine residues. For this purpose mutagenic primers 5′-GTGGGATCCATGGCAGCGAGCGAGATGGCTTGGAG-3′ and 5′-CTCAGCCATATCTGCTGCTGCTGCATGGATCCTCCCACA-3′ were used. (b) Tβ4(K16A) lacking the G-actin binding site. This mutant was obtained using mutagenic primers 5′-GATAAGTCGAAACGACGCAGCAGAGAGCAGGAAGAG-3′ and 5′-CTCTGGCTTCCTGCTGCTGCTGCTGCTGATCTATGC-3′. (c) Tβ4(K16A) with a dysfunctional nuclear localization signal sequence obtained by means of mutagenic primers 5′-AAATTCCGATAAGTCGACCTGAGAAGACAG-3′ and 5′-CTGTGTCTTCTCAGTGCAGCTTACG-3′. All Tβ4 mutants were produced using a QuickChange Site-directed Mutagenesis set (Stratagene), propagated in E. coli, purified with Wizard Midiprep (Promega), and sequenced to confirm the open reading frame.

Plasmids containing the EGFP-Tβ4 or EGFP-Tβ4 mutants were transfected into EA.hy 926 cells with Lipofectamine. Briefly, 5.0 μg of plasmid DNA and 5 μl of Lipofectamine solution were incubated for 45 min in 200 μl of Opti-MEM (Invitrogen), and then diluted with 800 μl of Opti-MEM. This solution was added to growing EA.hy 926 cells in 20% fetal bovine serum medium. Expression of the fusion construct was evaluated by confocal microscopy after 24 and 48 h.

**Real-time Quantitative RT-PCR**—To measure PAI-1 mRNA, EA.hy 926 cells transfected with pEGFP-N1-Tβ4, pEGFP-N1-Tβ4(AaSDKP/F4A), pEGFP-N1-Tβ4(K16A), and pEGFP-N1-Tβ4(K16A) were used for total RNA, extracted by the TRizol reagent method (Invitrogen) using a single-step purification protocol. RNA pellets were dissolved in water, and their concentrations and purity determined by spectrophotometer readings at 260 and 280 nm. Then, mRNAs for PAI-1 and β-actin expression were quantified by real-time RT-PCR using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) as we recently described (30). The following oligonucleotide primer pairs were used: 5′-TGCTGGTGAATGGCCTCTACT-3′ and 5′-GGGTTACATCAGGCAGCGAGCAG-3′ as well as 5′-CTCACCTTACAGGCAGCGAGCAG-3′ and 5′-GTGTGACAGCTTACGCTTGC-3′ specific for mRNAs of PAI-1 and β-actin, respectively. Relative gene expression levels were obtained using the ΔΔCt method (31). Amplification of specific transcripts were further confirmed by obtaining melting curve profiles. Similarly, mRNAs of Ku80, Tβ4, and von Willebrand factor were analyzed using appropriate oligonucleotide primer pairs.

**Tβ4-binding Proteins**—Recombinant Tβ4 was expressed in E. coli (BL21(DE3)pLysS) transformed with pRSETa-Tβ4. Harvested cells were centrifuged, resuspended in binding buffer (5 mM imidazole, 150 mM NaCl, 20 mM Tris-HCl pH 7.9), and sonicated on ice. Bacterial lysate was clarified by ultracentrifugation for an hour at 100,000 × g at 4 °C, filtered through a
0.45-μm syringe filter, and loaded onto a HisTrap column (5 ml, Amersham Biosciences) connected to an FPLC system (Amersham Biosciences). Proteins were eluted with a 5–500 mM imidazole gradient. The purity of recombinant Tβ4 was confirmed by reversed phase ultra performance liquid chromatography and mass spectrometry.

Recombinant Tβ4 containing a 12-residue His tag (MRGSHHHHHHGS) at its C terminus was biotinylated with freshly prepared p-diazobenzoyl biocytin according to the manufacturer’s protocol (Pierce). To search for Tβ4-binding proteins in endothelial cells, total cell extract, nuclear proteins, and the cellular membrane fraction were isolated from EA.hy 926 cells. To obtain the cell lysate, EA.hy 926 cells were extracted with lysis buffer (20 mM Tris-HCl, pH 8.3, containing 5% glycerol, 200 mM NaCl, 1% Triton X-100, 1 mM EDTA and protease inhibitor mixture) and cleared by centrifugation for 30 min at 12,000 × g at 4 °C. Crude nuclear extracts of EA.hy 926 cells were prepared essentially as described by Dignam et al. (32), except that modified buffer C was used during nuclear extraction (33). Membranes of EA.hy 926 cells were isolated as described by Thepparit and Smith (34) and solubilized in 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 1% Triton X-100 and protease inhibitors.

In typical experiments, the cell lysate, the extract of nuclear proteins or solubilized membranes were divided into two parts: into the first one biotinylated Tβ4 was added and incubated overnight at 4 °C and the second was used as a control without Tβ4, kept under the same conditions. Then, streptavidin-Sepharose beads were added to both samples and incubated for an hour at 4 °C. The beads were then washed exhaustively with PBS, resuspended in SDS-PAGE loading buffer (0.1 M Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 5% β-mercaptoethanol, 0.5% Coomassie Brilliant Blue R-250), boiled for 5 min, and separated by SDS-PAGE. Protein bands were visualized by staining with silver according to the method compatible with the analysis of proteins by mass spectrometry (35) and the selected bands subjected to in-gel digestion with trypsin.

**Construction of Ku80 Deletion Mutants and Tβ4 in the pCMV Vector—**To express Ku80 (the 80-kDa subunit of ATP-dependent DNA helicase II), total RNA was isolated from EA.hy 926 cells using TriPure reagent (Roche) and reverse transcribed using Moloney murine leukemia virus Reverse Transcriptase (Epicerent). The resulting DNA encoding the whole length gene of Ku80 was amplified with the following primer pair: 5’-TACCTCTAGAATTCGGATGTTGCGGTCCGGG-3’ and 5’-CTCTCAACAGCCGCGCTTATATGTGCTGCAA-3’. The amplification product was cloned into EcoRI and NotI sites of pCMV-Myc vector expressing the protein containing the N-terminal c-Myc epitope tag. Deletion mutants of Ku80 were constructed essentially as described for the insertion of the Ku80 coding sequence but using PCR products corresponding to its different fragments. Thus, pCMV-Myc vectors expressing Ku80-(1–249), Ku80-(1–460), Ku80-(1–580), and Ku80-(368–732) were constructed using the following primer pairs: 5’-GACGGGAAATTCGGATGTTGCGGTCCGGG-3’ and 5’-GATACAGGCGCGCCCTAGACGGGCCATG-3’; 5’-TACCTCAGGGATTCGGATTGCGGTCGGGG-3’ and 5’-CTACATCTGGCCGCGCCCTAGAACTGCAA-3’; 5’-TACCTCTAGAATTCGGATGTTGCGGTCCGGG-3’ and 5’-CTGATTATGCCTGGCGCGCTTCAACGTGCAA-3’; 5’-GACGGGAAATTCGGATGTTGCGGTCCGGG-3’ and 5’-CTGATTTGCTGGCGCGCGCTTCAACGTGCAA-3’, respectively.

Likewise, synthetic DNA coding Tβ4 sequence was amplified using the oligonucleotide primers pairs: 5’-GCAGCGGAAATTTCGGGATGTTGCGGTCGGGG-3’ and 5’-CTAACTAGCAGCGCGGTCCCTAGAAGCTAATCAA-3’. The resulting amplicon was inserted into EcoRI and NotI sites of pCMV-HA vector expressing the protein with HA epitope tag at the N terminus.

Co-immunoprecipitation Experiments—EA.hy 926 cells were transiently cotransfected with pCMV-HA-Tβ4 (4 μg) and either pCMV-Myc-Ku80 or one of the mutants (pCMV-Myc-Ku80-(1–249), pCMV-Myc-Ku80-(1–460), pCMV-Myc-Ku80-(1–580), pCMV-Myc-Ku80-(368–732)) (4 μg) using Lipofectamine and collected after 48 h in lysis buffer (PBS, 1 mM EDTA, pH 8.0, 0.5% Triton X-100). Cell lysates were then incubated with 2.5 μg of rabbit polyclonal antibody to Myc tag on a rotator overnight at 4 °C. Subsequently, 100 μl of Protein A/G-agarose bead slurry was added to each cell extract and the incubation continued for another 3 h. The beads were washed, suspended in SDS-PAGE loading buffer, and boiled for 5 min. Proteins released from the resin were separated by electrophoresis by 7% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and immunodetected by HA tag mouse monoclonal antibody. Immunoprecipitation of the Ku80-Tβ4 complex from endothelial cell extracts was performed as described previously (26).

**RNA Interference of Ku80—**For silencing the Ku80 gene expression a set of four synthetic siRNAs targeting the following sequences of Ku80 mRNA were used: Ku1, 5’-GCAUGGAGUGUAUUCACAA-3’ (position 374–392), Ku2, AACACUUCGUGUUUCAGUG (position 1821–1839), Ku3, 5’-CGAGUAAACCCCGCUCAAAA-3’ (position 1868–1886), and Ku4, 5’-GACGAGCGCUUUAAACACU-3’ (position 1986–2005).
siRNA silencing the human glyceraldehyde-3-phosphate dehydrogenase gene served as a positive control, with the following sense strand sequence: 5‘-UGGUUCAACUGUUCAAAUUU-3’. As negative control the functional non-targeting siRNA was used, containing 4 mismatches for any human, mouse, and rat gene. The sequence of its sense strand was 5‘-UAGCGACUA- AACACUCAAUU-3’. Synthetic siRNAs (200 pm) were transfected to 50–80% confluent EA.hy 926 cells with Lipofectamine (10 µl) according to the manufacturer’s instructions. After 24 h of incubation synthetic Tβ4 at 160 nM or tumor necrosis factor-α at 10 ng/ml was added to particular cell cultures with the silenced Ku80 gene and concurrently to non-transfected cells. The cells were incubated for another 24 h and then harvested with TriPure reagent for total RNA and protein isolation.

Subsequently, mRNA for PAI-1, glyceraldehyde-3-phosphate dehydrogenase, and Ku80 expression was quantified by real-time RT-PCR as described above. The following primer pair specific for Ku80 were 5′-GCAATTTAACAGT- TCC-3′ and 5′-GAGGGCTTCTCTTTGCTG-3′.

**Western Immunoblotting**—Protein isolated from EA.hy 926 cells transfected with vectors expressing Tβ4 and its mutants, Ku80 or siRNA, were extracted with TriPure reagent according to the manufacturer’s protocol. After extraction, pellets were dissolved in 1% SDS aqueous solution and the protein concentrations were measured with BCA Protein Assay Kit and equalized between samples with 1% SDS. Protein extracts were subjected to SDS-PAGE and electroblotted onto nitrocellulose filters. The filters were blocked with Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20 then immunodetected by anti-PAI-1 and anti-Ku80 or anti-Myc tag mouse monoclonal antibody. Immunodetection was accomplished using the enhanced chemiluminescence kit and Kodak BioMax Light Film (Eastman Kodak). Developed films were scanned and protein bands quantitated by Gel Doc 2000 Gel Documentation System (Bio-Rad).

**Confocal Microscopy**—For microscopic examination of EA.hy 926 cells transfected with pEGFP-N1-Tβ4 or its mutants, cells (5 x 10⁴ cells/ml) were plated on Permanox Coverslips in 8-well tissue culture chamber slides (NUNC) with detachable chambered upper structures. After 24 h incubation, they were fixed with ice-cold 3% formaldehyde in PBS for 20 min, washed 3 times with PBS, and incubated with blocking buffer (PBS containing 3% bovine serum albumin). They were also counterstained with TRITC-phallolidin or Hoechst 33258 (Molecular Probes) to visualize actin filaments or nuclei, respectively. Endogenous Tβ4 was detected in the untransfected cells with rabbit antibodies specific to Tβ4, followed by staining with secondary antibodies conjugated with fluorescein. The cells were then visualized using a helium/neon ion laser (543 nm excitation) and analyzed with MultiScan version 8.08 software. For intracellular probe visualization the confocal laser microscope Leica TCS SP2 system in the Laboratory of Confocal Microscopy in Nencki Institute of Experimental Biology was used. The series of the single 0.2-μm optical sections were collected. The image has been scanned at high resolution (×63 oil objective, 1.32 NA).

**High Content Forster Resonance Energy Transfer Screening Analysis**—Distribution of pEGFP-N1-Tβ4, pEGFP-N1-Tβ4 (KLKKTET/7A), and pEGFP-N1-Tβ4 (K16A) in membranes and nuclei of the transfected EA.hy 926 cells was evaluated by high content screening analysis using Cellomics ArrayScan V1 HCS Reader (Thermo Fisher Scientific, Pittsburgh, PA). ArrayScan consists of a high resolution optical system, multiple bandpass emission filter with matched single band excitation filters (XF57 or XF100, Omega Optical), a CCD camera with frame grabber, and proprietary applications software. In this assay, an excitation filter wheel and multiple bandpass emission filters are used to enable multichannel imaging of fluorescence from several fluorophores in the same cells. This system automatically locates, focuses, and exposes fields of cells within black-sided 96-well microtiter plates in a user-defined manner (36, 37). Briefly, 0.2 µg of plasmid DNA and 0.5 µl of Lipofectamine solution were incubated for 45 min in 50 µl of Opti-MEM (Invitrogen). This solution was added to EA.hy 926 cells growing on a 96-well plate in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. After 6 h the medium was changed and the cells were further incubated for 24 h. Then, the cells were washed twice with PBS and the staining of plasma membrane and nuclei was performed with the Image-LIVE Plasma Membrane and Nuclear Labeling Kit (Molecular Probes). The plasma membranes were stained with red fluorescent Alexa Fluor 594 wheat germ agglutinin at 5.0 µg/ml and the nuclei, with the blue fluorescent Hoechst 33342 at 1 µM. Finally, 100 µl of PBS was added into each well and the fluorescent images of the cells for three fluorophores were taken at the following wavelengths (excitation/emission; nm) of 591/618, 350/461, and 485/535 for Alexa Fluor 594 wheat germ agglutinin, Hoechst 3342, or GFP, respectively. Triple emission images were acquired for 30 fields in each well of the plate using the ×20 objective. The cells were analyzed using Cyto- plasm to Cell Membrane Translocation BioApplication and Compartmental Analysis BioApplication software according to the manufacturer’s instructions. Statistical analysis of the obtained data were performed using t test for independent samples.

**Statistical Analysis**—All values are expressed as mean ± S.E. compared with controls and among separate experiments. Paired and unpaired Student’s t tests were employed to determine the significance of changes. A p value <0.05 was considered statistically significant.

**RESULTS**

**Subcellular Localization of Tβ4 in Endothelial Cells**—In the course of our studies on the mechanism by which Tβ4 influences angiogenesis we examined its intracellular activity in endothelial cells. In preliminary experiments the intracellular distribution of Tβ4 and its mutants fused with GFP was evaluated by confocal microscopy. To produce a functional Tβ4-GFP fusion protein, GFP was attached to the C-terminal residue of the peptide. The rationale for this fusion strategy was that the C-terminal region does not specify any known function of Tβ4. This design avoided modifying the N terminus of Tβ4, which is important for interaction with potential receptors assumed to exist, based on the biological activity of its N-termi-
They were then fixed with paraformaldehyde and counterstained with TRITC-phalloidin to visualize actin filaments or nuclei, respectively. Panel c shows localization of GFP-tagged Tβ4 (green) in EA.hy 926 cells transiently transfected with 5 μg of pEGFP-N1-Tβ4. B, accumulation of GFP-tagged Tβ4 and Tβ4SDKP/4A at the cell-cell contact sites. EA.hy 926 cells were transiently transfected with 5 μg of pEGFP-N1-Tβ4 (panels a–c), pEGFP-N1-Tβ4SDKP/4A (panels d–f), pEGFP-N1-Tβ4KLKKTET/7A (panels g and h), and pEGFP-N1-Tβ4(K16A) (panels k–m); fixed with paraformaldehyde; and then counterstained with TRITC-phalloidin (red) or Hoechst 33258 (blue). GFP (green, a, d, g, and k), TRITC (red, b, e, h, and l), and Hoechst 33258 (blue, c, f, i, and m) signals were visualized directly by confocal microscopy. Average fluorescence derived from GFP-tagged Tβ4 or its mutants and actin filaments when merged revealed cellular regions with colocalization of both proteins (yellow, panels c and f). Arrows show accumulation of GFP-tagged Tβ4 and Tβ4SDKP/4A at the cell-cell contacts. These cells are representative of a number of cells analyzed during five separate experiments.

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FIGURE 1. Subcellular localization of GFP-tagged Tβ4 or its mutants and endogenous Tβ4 in endothelial cells. A, the control untransfected EA.hy 926 cells treated with specific antibodies to Tβ4 (panel a) or nonimmune rabbit IgG (panel b), followed by second antibodies conjugated with fluorescein. Control cells were then fixed with paraformaldehyde and counterstained with TRITC-phalloidin (red) and Hoechst 33258 (blue) to visualize actin filaments or nuclei, respectively (panel b). Panel c shows localization of GFP-tagged Tβ4 (green) in EA.hy 926 cells transiently transfected with 5 μg of pEGFP-N1-Tβ4. B, accumulation of GFP-tagged Tβ4 and Tβ4SDKP/4A at the cell-cell contact sites. EA.hy 926 cells were transiently transfected with 5 μg of pEGFP-N1-Tβ4 (panels a–c), pEGFP-N1-Tβ4SDKP/4A (panels d–f), pEGFP-N1-Tβ4KLKKTET/7A (panels g and h), and pEGFP-N1-Tβ4(K16A) (panels k–m); fixed with paraformaldehyde; and then counterstained with TRITC-phalloidin (red) or Hoechst 33258 (blue). GFP (green, a, d, g, and k), TRITC (red, b, e, h, and l), and Hoechst 33258 (blue, c, f, i, and m) signals were visualized directly by confocal microscopy. Average fluorescence derived from GFP-tagged Tβ4 or its mutants and actin filaments when merged revealed cellular regions with colocalization of both proteins (yellow, panels c and f). Arrows show accumulation of GFP-tagged Tβ4 and Tβ4SDKP/4A at the cell-cell contacts. These cells are representative of a number of cells analyzed during five separate experiments.

Additionally stained with TRITC-phalloidin and Hoechst 33258 (Fig. 1A, b, red and blue). Cells transfected with the GFP-tagged Tβ4 showed much higher expression of the fluorescently labeled intact Tβ4. Its distribution matched that of the endogenous Tβ4, showed much higher accumulation in cytoplasm and was close to the membranes (Fig. 1A, c, green). Endogenous Tβ4 similar to GFP-tagged Tβ4 colocalized with actin at the edges of cells (Fig. 1B, c, yellow). There were heavy accumulations of GFP-tagged Tβ4 and GFP-tagged Tβ4SDKP/4A close to the membranes, particularly at the cell-cell contact sites (Fig. 1B, a and c). Actin filament staining in the same cells was perhaps even more concentrated at the cell-cell contact sites (Fig. 1B, b), and overlapping staining of Tβ4 and actin seen as yellow was particularly evident at these sites. Besides the accumulations visualized within the cytoplasm, the data obtained also showed in many cases a punctate nuclear staining, indicating accumulation of intact Tβ4 within the cell nucleus. Within the nuclei there was a homogenous distribution of Tβ4 with the exception of regions of presumed nucleoli that were void of Tβ4. Similar to the wild-type Tβ4, overexpression of Tβ4SDKP/4A affected intracellular formation of actin filaments that could be found predominantly in the periphery of cells, directly under the cell membrane (Fig. 1B, d, e, and f).

Mutants Tβ4KLKKTET/7A and Tβ4K16A differ significantly in their cytoplasmic distribution from that of the wild-type Tβ4 (Fig. 1B, g, h, i and k, l, m, respectively): their fluorescence is more punctuated and they do not accumulate in the submembrane regions. In contrast to wild-type Tβ4 and Tβ4SDKP/4A cells transfected with Tβ4KLKKTET/7A showed a well-developed system of the actin filament network typical of adhering and spread cells. This indicates that the mutant lacked the actin binding site and thus did not interfere with actin polymerization. It is noteworthy that localization of Tβ4 and its mutants in endothelial cells shown in Figs. 1 and 2 was observed in a number of cells (from 47 to 95) analyzed by confocal microscopy during five separate experiments. These data indicate that attachment of EGF to the polypeptide chain of Tβ4 did not influence its primary function, which is to interact with G-ac- tin. To examine whether the point mutation within the nuclear localization sequence K16A reduced accumulation of this mutant within the cell nucleus, optical sections of cells transfected with pEGFP-N1-Tβ4 or pEGFP-N1-Tβ4K16A were

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Intracellular Expression of Tβ4 and PAI-1—In the next experiments we tested whether increased intracellular levels of Tβ4 or its functionally modified mutants affect the mechanisms by which expression of the PAI-1 gene is controlled. For this purpose, PAI-1 expression was evaluated at the level of both mRNA and protein synthesis in EA.hy 926 cells transfected with Tβ4 and its mutants. Because transfection efficiency differed from one construct to another, expression of PAI-1 mRNA was normalized and the plotted values correspond to those that would be obtained after transfection of all cells. Fig. 3A shows that transfection with pEGFP-N1-Tβ4 and pEGFP-N1-Tβ4(AcsDKP/TA4) resulted in inducing the PAI-1 gene almost to the same extent as is demonstrated by real-time PCR analysis of PAI-1 mRNA. Thus, depletion of the N-terminal tetrapeptide AcSDKP did not influence the ability of Tβ4 to up-regulate PAI-1 expression. Interestingly, elimination of the actin binding sequence KLKKTET or damage of the nuclear localization sequence by a point mutation (K16A) only partially reduced the ability of such Tβ4 mutants to up-regulate PAI-1 expression. When tested at the protein synthesis level, as shown by immunoblotting with anti-PAI-1 antibodies followed by scanning, increased intracellular concentrations of Tβ4 and all its mutants also induced PAI-1 expression in transfected cells (Fig. 3B).

Tβ4-binding Proteins in Endothelial Cells—To investigate the potential mechanisms through which Tβ4 might be influencing endothelial cell migration and survival events, we then searched for Tβ4 interacting proteins within the cells. For this purpose, the recombinant Tβ4 was biotinylated with p-diazo-benzoic biocytin reagent in such a way that exclusively His residues were then conjugated to the biotin ligase, and the biotinylated Tβ4 was used for screening analysis using a Cellomics ArrayScan VTI HCS Reader. Due to specific staining of membranes and nuclei with Alexa Fluor 594 wheat germ agglutinin and Hoechst 33342, respectively, this system automatically locates, focuses, and exposes labeled fields of cells. Then, location, translocation, and quantification of Tβ4 and its mutants containing yellow GFP showing the optimized spectral overlap with both dyes are accomplished by Forster resonance energy transfer. Fig. 2, C and D, show that Tβ4 and its mutants are equally localized close to the membrane but their nuclear accumulation is significantly different. Both, Tβ4(KLKKTET/TA4) and Tβ4(K16A) occur in much lower concentrations in nuclei than intact Tβ4 indicating that their nuclear uptake is impaired.
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Increased intracellular expression of Tβ4 induces the PAI-1 gene in endothelial cells. EA.hy 926 cells were transiently transfected with 5 μg of pEGFP-N1-Tβ4, pEGFP-N1-Tβ4(KLKKTET/7A), or pEGFP-N1-Tβ4(K16A), and PAI-1 expression was evaluated at the level of both mRNA and protein synthesis. A, changes in mRNA levels in transfected EA.hy 926 cells. In these experiments, total cellular RNA was extracted from cells 48 h after transfection with Tβ4 and its mutants, and PAI-1 mRNA was quantified by real-time PCR. This experiment was repeated three times with similar results. B, transfection efficiency differed from one construct to another, expression of PAI-1 mRNA was normalized to 100% of transfection efficiency. B, PAI-1 protein in extracts of the same cells evaluated by Western immunoblotting (inset). Immunodetection of PAI-1 was accomplished using an enhanced chemiluminescence kit, and films were scanned and protein bands quantitated using the Gel Doc 2000 gel documentation system (Bio-Rad). To quantify the densitometric scans, the background was subtracted and the area for each protein peak was determined. Data were obtained from three separate experiments and normalized to 100% of transfection efficiency.

Ku80 interacts with Tβ4 in endothelial cells. Biotinylated Tβ4 was incubated with extracts of cells (A), isolated membranes (B), or nuclear proteins (C), and then aliquots of streptavidin-Sepharose suspension were added. After incubation, the resin was washed, and Tβ4-interacting proteins were dissociated with the SDS sample buffer and analyzed by SDS-PAGE. In parallel, samples of starting material (SM) and proteins eluted from streptavidin-Sepharose in the absence of biotinylated Tβ4 were also analyzed. D, the presence of Ku80 was detected by Western immunoblotting in immunoprecipitates obtained from endothelial cells with antibodies specific to Tβ4. In parallel, samples of cell extract were incubated with control normal IgG. Then, proteins precipitated with anti-Tβ4, normal IgG, and those present in starting cell extracts were separated by SDS-PAGE and blotted with anti-Ku80 antibodies.

Among them, a protein with molecular mass of 80 kDa was consistently found to be bound to Tβ4 regardless of the starting material used, namely the total cell lysate, solubilized cell membranes, or the extract of nuclear proteins. This protein was absent in the control samples missing the biotinylated Tβ4 (Fig. 4, A–C). It was identified with confidence, by peptide sequencing and peptide mass fingerprinting using an electrospray (ESI-Q-TOF-Micromass) spectrometer, to be ATP-dependent DNA helicase II, specifically its Ku80 subunit (Table 1). In addition to some other cellular proteins, large amounts of actin were also detected to be associated with both types of resin, i.e. the control resin missing Tβ4 and the one containing immobilized Tβ4. We then investigated whether Tβ4 can interact in vivo with Ku80 in endothelial cells. To identify Ku80-Tβ4 complexes consisting of endogenous proteins, we performed co-immunoprecipitation experiments using extracts of untransfected endothelial cells. Proteins were precipitated with specific rabbit polyclonal antibodies to Tβ4, and immunoprecipitates were washed and separated by SDS-PAGE. Ku80 bound to Tβ4 was identified by Western immunoblotting using antibodies specific to Ku80 or nonimmune rabbit sera (Fig. 4D).

To narrow down localization of the Ku80 binding site for Tβ4 and prove the specificity of this interaction, the Ku80 deletion mutants were constructed and expressed in pCMV-Myc vector (Fig. 5A). As detected by immunoblotting with specific antibodies to Myc tag, EA.hy 926 cells after transfection with these constructs showed almost the same expression of Ku80 fragments, namely Ku80(1–249), Ku80(1–460), Ku80(1–580), Ku80(368–732), or intact molecule (Fig. 5B). EA.hy 926 cells were then cotransfected with pCMV-HA-Tβ4 and pCMV-Myc-Ku80 followed by immunoprecipitation performed using rabbit anti-HA antibodies. Fig. 5C shows the presence of Ku80 in the co-precipitating proteins as evidenced by immunoblotting with anti-Myc monoclonal antibody, thus confirming that Ku80 can form the complex with Tβ4 in endothelial cells. Fig. 5D shows that in addition to intact Ku80, only Ku80(1–580) and Ku80(368–732) form a complex with Tβ4...
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FIGURE 5. Binding of Ku80 deletion mutants to Tβ4. A, schematic representation of the Ku80 deletion mutants. All constructs contained COOH-terminal Myc tag. Their expression in endothelial cells transfected with corresponding constructs detected by specific antibodies to Myc tag is presented in B, Binding of the Ku80 deletion mutants to Tβ4 was tested after cotransfection of EA.hy 926 cells with pcmv-HA-Tβ4 and the vectors expressing corresponding Ku80 fragments. In C, the formation of the Ku80-Tβ4 complex is evidenced in the reversed system, i.e. when immunoprecipitation (IP) is performed using anti-HA antibodies and immunoblotting with anti-Myc antibodies, respectively. D, co-immunoprecipitation of Tβ4 with Ku80, Ku80-(1–580), and Ku80-(368–732) by anti-Myc antibodies, followed by immunoblotting with monoclonal anti-HA. E, changes in mRNA levels in EA.hy 926 cells transfected with pcmv-Myc-Ku80 or one of the mutants (pcmv-Myc-Ku80-(1–249), pcmv-Myc-Ku80-(1–460), pcmv-Myc-Ku80-(1–580), and pcmv-Myc-Ku80-(368–732)). In these experiments, total cellular RNA was extracted from cells 48 h after transfection, and PAI-1 mRNA was quantified by real-time PCR. This experiment was repeated three times with similar results. Because transfection efficiency differed from one construct to another, expression of PAI-1 mRNA was normalized to 100% of transfection efficiency. F shows PAI-1 protein in extracts of the same cells evaluated by Western immunoblotting (inset figure). Immunodetection of PAI-1 was accomplished as described in the legend to Fig. 3B. Data were obtained from three separate experiments and normalized to 100% of transfection efficiency.

and can be immunoprecipitated from endothelial cells cotransfected with pcmv-HA-Tβ4 and pcmv-Myc vector expressing Ku80 or its mutants. This indicates that the C-terminal arm domain of Ku80 is directly involved in interaction with Tβ4 and its deletion destroys Tβ4 binding site. To further demonstrate the role of Ku80 in regulation of PAI-1 expression, in the next experiments PAI-1 expression was evaluated at the level of both mRNA and protein synthesis in EA.hy 926 cells transfected with pcmv-Myc-Ku80 or one of the mutants (pcmv-Myc-Ku80-(1–249), pcmv-Myc-Ku80-(1–460), pcmv-Myc-Ku80-(1–580), and pcmv-Myc-Ku80-(368–732)). Data showed in Fig. 5, E and F, were normalized using corresponding transfection efficiency. Only transfection of cells with pcmv-Myc-Ku80 or pcmv-Myc-Ku80-(1–580) resulted in up-regulation of PAI-1 expression detected at the level of both mRNA and protein synthesis. Because all other mutants, including Ku80-(368–732), did not influence PAI-1 expression in the transfected cells it indicates that binding of Tβ4 to the C-terminal arm domain (Ku80-(460–580) is not sufficient to stimulate PAI-1 expression but to show such activity it requires the presence of the α/β domain (Ku80-(1–249)).

Down-regulation of Ku80 by siRNA and PAI-1 Expression—To explore the impact of Ku80 on PAI-1 expression in Tβ4-activated endothelial cells, we employed specific siRNA as a means of depleting this protein in EA.hy 926 cells. For this purpose, siRNAs complementary to four regions of Ku80 were generated, and their efficacy in down-regulating Ku80 in endothelial cells was analyzed. Fig. 6, A and B, show control experiments demonstrating that all four siRNAs to Ku80 mRNA and their equimolar mixture reduced expression of the targeted protein

TABLE 1

Tβ4-interacting proteins identified in subcellular fractions of endothelial cells

The subcellular fractions were isolated from HUVECs as described under “Experimental Procedures.” The biotinylated Tβ4 was added to the total cell lysate, the cell membrane lysate, or nuclear protein fraction. After incubation, the biotinylated Tβ4 and bound proteins were separated using streptavidin immobilized on Sepharose. Samples of proteins bound were separated by SDS-PAGE followed by sequencing of protein bands selectively bound to Tβ4 (Fig. 4). Protein bands were identified after silver staining and analyzed by sequencing as described under “Experimental Procedures.”

| Cell fraction | Band | g| Protein | Mass | Peptides matched | Total score |
|---------------|------|---|---------|------|-----------------|-------------|
| Cell lysate   | 1    | 10863945 | ATP-dependent DNA helicase II (Ku80) | 83,222 | 14 | 812 |
| Cell membranes| 1 | 10863945 | ATP-dependent DNA helicase II (Ku80) | 83,222 | 17 | 1,000 |
| Nuclear proteins | 1 | 10863945 | ATP-dependent DNA helicase II (Ku80) | 83,222 | 10 | 560 |

a gi accession numbers of the identified proteins in the protein data base.
b Predicted molecular masses of identified proteins.
c Numbers of peptides identified from the amino acid sequences of assigned proteins.
d Total probability scores from the Mascot search for identified proteins.
in endothelial cells to almost undetectable levels within 72 h of transfection, as demonstrated at the level of both mRNA (panel A) and protein (panel B) synthesis analyzed by real-time RT-PCR and Western blotting, respectively. Control siRNA when used at the same concentration did not have any effect on Ku80 expression. Three days after transfection of oligos, when efficient knockdown of Ku80 was observed, cells were collected and PAI-1 expression was tested at the level of mRNA by real-time PCR, or protein synthesis by Western immunoblotting (Fig. 6, C and D). In cells treated with control siRNA, Tβ4 alone increased PAI-1 to the level comparable with those observed in the untreated cells (not shown). Expression of von Willebrand factor and Tβ4 analyzed in the same samples was not affected when analyzed using both RT-PCR (panel E) or Western blotting (panel F). Thus, these data show that inhibition of Ku80 expression resulted in specific abolishment of the PAI-1 signal determined at the level of its mRNA and PAI-1 antigen synthesis. Furthermore, cells depleted of Ku80 with siRNA failed to elicit a robust increase in PAI-1 typical for the untreated cells. Taken together these data suggest that Ku80, after formation of complexes with Tβ4, is involved in regulation of PAI-1 expression in endothelial cells induced by Tβ4.

**DISCUSSION**

A number of studies showed that Tβ4 exhibits biological functions that are important for angiogenesis, wound healing, and the regeneration and remodeling of injured tissues (for review, see Ref. 38). Up to now two bioactive fragments of Tβ4 have been identified, namely the N-terminal tetrapeptides AcSDKP (39) and LKKTET, derived from its central actin-binding domain (40). Although there are observations revealing that Tβ4 and its fragments affect cellular processes by activation of several signaling pathways and induction of changes in cell properties necessary for both migration and survival, not much is known about the mechanisms by which they express their functions.

The data presented here demonstrate that increased intracellular expression of Tβ4 is necessary and sufficient to initiate PAI-1 gene expression in Tβ4-activated endothelial cells. This conclusion is based on the constitutively enhanced expression of Tβ4 in EA.hy 926 cells prior to the induction of the PAI-1 gene by extracellularly added Tβ4. Interestingly, the mechanism of this intracellularly active Tβ4 only partially depends on its ability to bind G-actin or the presence of other bioactive regions in the Tβ4 molecule such as the N-terminal tetrapeptide and the nuclear localization sequence. This conclusion is based on the following observations: (a) substitution of the N-terminal tetrapeptide with four alanine residues did not have any influence on stimulation of PAI-1 expression in the transfected cells. (b) Mutation of bioactive motifs responsible for binding G-actin and entering the nucleus selectively damaged their functions. Neither mutation abolished the ability of such mutants to up-regulate PAI-1 expression in the transfected cells although they showed an ~40% decrease in activation of the PAI-1 gene. (c) Tβ4 and Tβ4(AcSDKP7T4A) showed the same levels of overexpression and a similar distribution in the transfected cells as evaluated by confocal microscopy. They were densely accumulated close to the membranes, particularly at cell-cell contact sites.

One of the notable findings of this study is the identification of previously unknown Tβ4-interacting proteins in endothelial cells, especially the Ku80 subunit of ATP-dependent DNA helicase II, which was consistently associated with the biotinylated Tβ4. Ku80 specifically interacted with Tβ4 when total cell lysate, isolated nuclear proteins, or plasma membranes were taken as a starting material. Moreover, Ku80 and Tβ4 con-
sistantly immunoprecipitated in a common complex from extracts of both control endothelial cells and the cells that were cotransfected with Ku80 and Tß4 fused with peptide tags. Furthermore, co-immunoprecipitation experiments with Ku80 deletion mutants showed that Tß4 directly interacts with the C-terminal arm domain of Ku80 but to stimulate PAI-1 expression it requires both the C-terminal arm and the α/ß domains to be in close proximity. Interestingly, down-regulation of Ku80 expression with siRNA in endothelial cells resulted in a dramatic reduction of PAI-1 synthesis, indicating that Ku80 is involved in signaling initiated by Tß4 that leads to activation of PAI-1 gene expression by a still unknown mechanism.

The Ku proteins were originally identified as autoantigens, recognized by the sera of patients with autoimmune diseases such as systemic lupus erythematosus and scleroderma (41). The two Ku proteins, Ku70 and Ku80, have been well demonstrated to dimerize and function in repair of DNA double strand breaks, DNA telomere length maintenance, transcription regulation, and V(D)J recombination (42–45). Accordingly, Ku is thought to play a crucial role in maintenance of chromosomal integrity and cell survival (for review, see Ref. 46). Observations through various experimental models indicate that Ku may act as either a tumor suppressor or an oncoprotein. Although they are predominantly nuclear proteins and primarily can be found in the transcriptionally active regions of the nucleus, recent studies showed that Ku proteins are also expressed in the cytoplasm, on the cell surface, and in the extracellular matrix (47, 48). Ku is a component of the DNA-PK complex in membrane rafts of mammalian cells (49) and its membrane expression can be induced at hypoxia. Interestingly, it mediates adhesion of cells to fibronectin (48, 50, 51), which indicates its role as an adhesion receptor (52). Recently, Ku80 was also identified to be a coreceptor for human parvovirus B19 infection (53). It appears that both Ku80 and signal transduction are coupled (49, 54). Furthermore, Ku can interact with matrix metalloproteinase 9 at the cell surface of highly invasive hematopoietic cells of normal and tumor cell origin, and Ku80/metalloproteinase-9 interaction at the cell membrane may result in contributing to invasion by tumor cells through regulation of extracellular matrix remodeling (48). Ku proteins can also function as transcription factors and bind in a sequence-specific manner to promoter elements (55). For example, Ku86 binds to the promoter and regulates the genes of the heat shock proteins, glucose-regulated peptide 78, grp94 (56), and S100A9 gene expression (57).

At present the mechanism by which Ku80 contributes to Tß4-induced PAI-1 gene regulation is unclear. Ku proteins have been shown to regulate numerous intracellular functions, suggesting that they might be active at multiple locations within the cell. Thus, Ku80 may function at different steps of signal transduction induced by Tß4 and leading to up-regulation of PAI-1 expression. Because it is found in membrane rafts it may play a role of Tß4 coreceptor and coactivator. Ku80 has a nuclear localization sequence and, consistent with data presented in Fig. 3D, may facilitate transport of Tß4 into the nucleus. Finally, Ku80 can be a cofactor that binds to the PAI-1 promoter and potentiates its Tß4-induced activation.

To sum up, in these studies we have provided evidence that increased intracellular expression of Tß4 leads to induction of the PAI-1 gene in Tß4-activated endothelial cells. This effect involves complex formation with Ku80, which functions as a novel receptor for Tß4 and provides an alternative mechanism of the intracellular activity of Tß4 different from sequestering of G-actin.

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