Phosphorylation of the human-transforming-growth-factor- β -binding protein endoglin

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Endoglin is an homodimeric membrane antigen with capacity to bind transforming growth factor- β (TGF- β). Phosphorylation of human endoglin was demonstrated in endothelial cells as well as in mouse fibroblast transfectants expressing two isoforms, L-endoglin or S-endoglin, with distinct cytoplasmic domains. The extent of L-endoglin phosphorylation was found to be 8-fold higher than that of S-endoglin, and phosphopeptide analyses

revealed at least three different phosphorylation sites for L-endoglin, whereas S-endoglin produces only one phosphopeptide. The immunoprecipitated L-endoglin was found to be phosphorylated mainly on serine, and, to a minor extent, on threonine, residues. Treatment of the cells with TGF- β 1 or the protein kinase C inhibitor H-7 resulted in a reduction of the levels of endoglin phosphorylation.

INTRODUCTION

Endoglin, also known as 'CD105' (Endothelial Section, Fifth International Conference on Human Leukocyte Differentiation Antigens, Boston, U.S.A., November 3–7, 1993), is a 180 kDa homodimeric membrane glycoprotein expressed by human endothelial cells [1], macrophages [2,3], erythroid precursors [4], syncytiotrophoblasts of term placenta [5] and fibroblasts [1]. The gene encoding endoglin has been localized to human chromosome 9 [6]. Recently it has been demonstrated that endoglin binds TGF- β 1 and TGF- β 3 with high affinity (K_D 50 pM) in human endothelial cells [7]. Two different isoforms, L-endoglin and S-endoglin, with capacity to bind TGF- β , but differing in the amino acid composition of their cytoplasmic tails, have been characterized [8,9]. β -Glycan and endoglin belong to a class of TGF- β receptors (type III receptors) that may function as regulators of TGF- β access to the signalling heteromeric kinase receptor complex formed by TGF- β receptor components I and II [10–12]. Interestingly, β -glycan and the predominant isoform, L-endoglin, share a region of high identity in the cytoplasmic tail with a high content (40 %) of Ser/Thr residues [8,13–15], which suggests that this domain might undergo phosphorylation by a Ser/Thr kinase. Here we have characterized the phosphorylation of human endoglin.

MATERIALS AND METHODS

Cells

Human umbilical-vein endothelial cells (HUVEC) were isolated from term umbilical-cord veins by collagenase perfusion as described in [16]. HUVEC were maintained in RPMI medium containing 20 % (v/v) fetal-calf serum, 50 μ g/ml endothelial cell growth supplement (Collaborative Research) and 100 μ g/ml of heparin (Sigma). Phosphorylation assays were performed using cells at passages 2–4. Mouse fibroblast transfectants expressing human L-endoglin or S-endoglin were generated by cotransfection of full-length cDNA encoding L-endoglin or S-endoglin in pcEXV and psV2neo [9]. After selection in RPMI medium

containing 10 % fetal-calf serum and 400 μ g/ml of the antibiotic G418 (Sigma), endoglin-positive clones were isolated by flow-cytometry analysis. Endoglin-negative clones were selected as mock transfectants. To remove the cells from the culture flasks, HUVEC or mouse transfectant monolayers were treated with trypsin for 3 min at room temperature and washed with RPMI containing 10 % fetal-calf serum at 4 °C.

Phosphorylation studies

Confluent monolayers of HUVEC and transfectant mouse fibroblasts from 75 cm² flasks were preincubated for 30 min at 37 °C in phosphate-free RPMI medium containing dialysed 10 % fetal-calf serum. After preincubation, the medium was removed and fresh medium containing 100 μ Ci/ml of [³²P]orthophosphate (ICN Biomedicals) was added. Cells were metabolically labelled at 37 °C under a 5 % CO₂ atmosphere for 4 h. In some experiments, labelling of endoglin transfectants with [³²P]orthophosphate was performed as described above, but in the presence of 10 ng/ml of TGF- β 1 (British Biotechnology). Experiments with the protein kinase C (PKC) inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) (Sigma) were carried out by culturing the cells at 10 μ M or 100 μ M of H7 for 30 min, followed by the [³²P]orthophosphate labelling, also in the presence of H7. At these concentrations, the viability of the cells was over 95 %.

Metabolic labelling with [³⁵S]methionine/cysteine

Confluent monolayers of transfectant mouse fibroblasts from 75 cm² flasks were preincubated for 30 min at 37 °C in RPMI methionine/cysteine-free medium containing dialysed 10 % fetal-calf serum. After preincubation, the medium was removed and fresh medium, containing 100 μ Ci/ml of [³⁵S]methionine/cysteine (Trans³⁵S-label; ICN Biomedicals), was added. Cells were metabolically labelled for 14 h at 37 °C under a 5 % CO₂ atmosphere.

Abbreviations used: HUVEC, human umbilical-vein endothelial cells; TGF- β , transforming growth factor- β ; PKC, protein kinase C; mAb, monoclonal antibody.

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Figure 1 Phosphorylation of endoglin

(a) Immunoprecipitation analysis of endoglin in endothelial cells. Human endothelial cells were metabolically labelled with [³²P]orthophosphate, lysed, and immunoprecipitated with 44G4 (anti-endoglin), 8E11 (anti-endoglin) or HC1/1 (anti-CD11c) mAb. The HC1/1 mAb was used as a negative control. Samples were subjected to SDS/PAGE and autoradiography. (b) Immunoprecipitation analysis of L-endoglin and S-endoglin. Mouse fibroblast transfectants expressing either L-endoglin or S-endoglin were metabolically labelled with either [³²P]orthophosphate or [³⁵S]methionine, lysed, and immunoprecipitated with 44G4 (anti-endoglin). Samples were subjected to SDS/PAGE and autoradiography. Bands of 170 kDa and 160 kDa corresponding to L-endoglin and S-endoglin respectively are indicated. Open arrowheads (▷) indicate an additional 100 kDa band specifically immunoprecipitated from L-endoglin transfectants. (c) Phosphopeptide analysis of endoglin isoforms. L-endoglin (i) and S-endoglin (ii) were immunoprecipitated from [³²P]orthophosphate-labelled transfectants and digested with the endoproteinase Glu-C from *S. aureus* and with the proline-specific endopeptidase from *F. meningosepticum*. Phosphopeptides were separated by high-voltage thin-layer electrophoresis. The origin of the samples and the separated peptides were indicated by ▼ and Δ respectively. (d) Phosphoamino acid analysis of L-endoglin immunoprecipitated from [³²P]orthophosphate-labelled transfectants. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

Immunoprecipitation studies

Metabolically labelled cells were lysed in PBS, pH 7.4, containing 1% Triton X-100, 1 mM phenylmethanesulphonyl fluoride, 1 mM orthovanadate, 1 mM sodium molybdate and 1 mM NaF (lysis buffer) for 15 min at 4 °C. The lysates were centrifuged for 20 min at 12000 *g* and pre-cleared for 2 h with protein G coupled to Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) at 4 °C. Specific immunoprecipitations of the pre-cleared lysates were carried out in the presence of 10 µg/ml of the monoclonal antibodies (mAb) 8E11 (anti-endoglin) of the IgM class [2], 44G4 (anti-endoglin) of the IgG1 subclass [1] and HC1/1 (anti-CD11c) of the IgG1 subclass, using Protein G coupled to Sepharose [2]. After incubation at 4 °C for 4 h, immunoprecipitates were isolated by centrifugation, washed twice with lysis buffer at 4 °C and then subjected to SDS/PAGE on a 6%-acrylamide gel under non-reducing conditions, followed by autoradiography. The amount of endoglin radiolabel was quantified with a

PhosphorImager 410A and ImageQuant software (Molecular Dynamics).

Phosphopeptide analysis

³²P-Labelled L-endoglin or S-endoglin were immunoprecipitated from mouse transfectants and electrophoresed on a 6%-acrylamide gel. Endoglin bands were localized by autoradiography, cut out, and the protein was eluted from the gel as described in [18]. Proteolytic digestions were carried out first with endoproteinase Glu-C from *Staphylococcus aureus* V8 (EC 3.4.21.19; Boehringer Mannheim, Barcelona, Spain) in 0.1 M NH₄HCO₃, pH 7.4, overnight at 37 °C, followed by incubation with the proline-specific endopeptidase from *Flavobacterium meningosepticum* (EC 3.4.21.26; ICN Biomedicals) in 0.1 M phosphate buffer, pH 7.0, for 3 h at 30 °C. Phosphopeptides were separated by thin-layer electrophoresis in 10 g/l of (NH₄)₂CO₃, pH 8.9, for 30 min at 1 kV as described in [18]. Radiolabelled peptides were

detected on the plate with a PhosphorImager 410A and ImageQuant software (Molecular Dynamics).

Phosphoamino acid analysis

^{32}P -labelled L-endoglin was immunoprecipitated from mouse transfectants as described above, electrophoresed on a 6%-acrylamide gel and electroblotted on to nylon membranes (Hybond-N; Amersham International). The endoglin nylon band localized by autoradiography was cut out and directly digested in 200 μl of 6 M HCl at 110 $^{\circ}\text{C}$ for 4 h. Phosphoamino acids were analysed by two-dimensional thin-layer electrophoresis as previously described [18].

Flow cytometry

Cells (5×10^5) were incubated with the mAb 44G4 at 10 $\mu\text{g}/\text{ml}$ for 30 min at 4 $^{\circ}\text{C}$. After two washes with PBS containing 0.1% BSA and 0.04% NaN_3 , fluorescein isothiocyanate-labelled F(ab')₂ rabbit anti-mouse IgG (Dakopatts) was added and incubation proceeded for an additional period of 30 min at 4 $^{\circ}\text{C}$. Finally, cells were washed twice and their fluorescence was estimated with an EPICS-CS instrument (Coulter Científica, Móstoles, Spain) using logarithmic amplifiers.

RESULTS AND DISCUSSION

Human endoglin is constitutively phosphorylated

To analyse the phosphorylation status of endoglin, we used endothelial cells where endoglin is highly expressed [1]. These cells were metabolically labelled with [^{32}P]orthophosphate and analysed by immunoprecipitation (Figure 1a). A phosphorylated protein of 170 kDa was specifically immunoprecipitated with two different monoclonal antibodies to endoglin. Recently, two isoforms of endoglin with distinct cytoplasmic domains have been identified [9]. The predominant form is L-endoglin, which contains 47 amino acids in the cytoplasmic tail, rather than S-endoglin, which has 14 residues. To analyse the phosphorylation status of these isoforms, we carried out metabolic labelling of mouse fibroblast transfectants expressing either L-endoglin or S-endoglin. Immunoprecipitation studies revealed a heavily phosphorylated 170 kDa band of L-endoglin, which contrasted with a weakly labelled 160 kDa band corresponding to S-endoglin (Figure 1b). The phosphorylation of L-endoglin was found to be approx. 8-fold greater than that of S-endoglin, suggesting the existence of multiple phosphorylation sites in the long isoform. This result is in agreement with the presence in the cytoplasmic domain of 19 Ser/Thr residues in L-endoglin and 2 Ser/Thr residues in S-endoglin. As a control, immunoprecipitations from ^{35}S -labelled transfectants showed similar labelling of L-endoglin and S-endoglin (Figure 1b). On the other hand, a polypeptide of approx. 100 kDa was specifically immunoprecipitated from ^{32}P - or ^{35}S -labelled L-endoglin transfectants ($\triangleright$, Figure 1b). It will be of interest to analyse whether this band corresponds to a heteromeric association with the type II receptor, as reported for β -glycan [11]. Further support for multiple phosphorylation sites of L-endoglin was obtained by phosphopeptide analysis (Figure 1c). Thus digestions of the phosphorylated endoglin isoforms with glutamine- and proline-specific proteinases revealed at least three different phosphorylation sites for L-endoglin against only one for S-endoglin.

Since L-endoglin is the predominant form of endoglin in endothelial cells [9], we studied its phosphorylation in more detail. Phosphoamino acid analysis of ^{32}P -labelled L-endoglin

Figure 2 Effect of TGF- β and H-7 on endoglin phosphorylation

(a) Immunoprecipitation analysis. Mouse fibroblast transfectants expressing L-endoglin were metabolically labelled with [^{32}P]orthophosphate in either the absence or the presence of TGF- β_1 (10 ng/ml) or the PKC inhibitor H-7 (10 μM or 100 μM). Cells were lysed, then immunoprecipitated with 44G4 (anti-endoglin) mAb. Samples were subjected to SDS/PAGE and autoradiography. The radioactivity associated with the endoglin bands was quantified in the autoradiographs by using a PhosphorImager instrument (Molecular Dynamics), and the values obtained were plotted as percentage of the amount present in the absence of treatment. The results are means and S.D.s for three different experiments. Representative autoradiographs are shown. (b) Flow-cytometric analysis. Mouse fibroblast transfectants expressing L-endoglin were incubated in the absence or presence of TGF- β_1 (10 ng/ml) or the PKC inhibitor H-7 (100 μM) as described in the Materials and methods section. Then cells were stained for indirect immunofluorescence with the mAb 44G4 (anti-endoglin). Control stainings of endoglin⁻ mock transfectants are also shown. Numbers at the upper-right corner indicate the mean fluorescence intensity of the endoglin-positive transfectants. The broken vertical line represents the limit of specific fluorescence. The result obtained at 10 μM H-7 was found to be similar to that shown here at 100 μM H-7. This is a representative experiment of another two that gave similar results.

indicated that serine, and to a minor extent threonine, residues were phosphorylated (Figure 1d). We could not detect any signal corresponding to phosphotyrosine. Furthermore, no specific

recognition of either L-endoglin or S-endoglin was achieved by Western-blot analysis using an anti-phosphotyrosine antibody (result not shown).

Effect of TGF- β and the PKC inhibitor H7 on the phosphorylation of endoglin

The signalling TGF- β receptor types I and II form a ligand-induced heteromeric complex with β -glycan [11] and have been shown to contain serine/threonine kinase activities in their intracellular domains [19,20]. To analyse whether the signalling induced by TGF- β affects phosphorylation of endoglin, we carried out [32 P]orthophosphate labelling in the presence of ligand (Figure 2a). Addition of TGF- β 1 was found to decrease significantly the phosphorylation level of L-endoglin. As a control, the level of endoglin surface expression was found to be unaffected in the presence of TGF- β 1 (Figure 2b), excluding a ligand-induced internalization of endoglin. This inhibitory effect on L-endoglin phosphorylation was reproduced in three different experiments and was also observed with S-endoglin (results not shown). Whether the mechanism of this TGF- β 1-induced inhibition of phosphorylation is shared with that reported for the retinoblastoma protein [21] remains to be determined.

A computer search for consensus sequences of phosphorylation (Database PROSITE; GCG Software Genetics Computer Group) revealed that the L-endoglin cytoplasmic domain contains one potential phosphorylation site for PKC (serine-621; RXXSKR). To analyse the possible involvement of this kinase, we carried out phosphorylation studies in the presence of the PKC inhibitor H-7 (Figure 2a). Addition of H-7 clearly inhibited the phosphorylation of endoglin at two different concentrations, whereas surface expression of endoglin was found to be unaffected (Figure 2b).

The cytoplasmic domains of β -glycan and L-endoglin are highly conserved in human and rat [8,13–15], implying a common important function for this domain. We find that human L-endoglin is constitutively phosphorylated on serine and threonine residues, suggesting a role in signalling and/or cytoplasmic association with other proteins. Our results do not support a TGF- β -dependent participation by the Ser/Thr kinase activity of the signalling receptors I and II, but they do not exclude their involvement in the absence of ligand. Since the phosphorylation of endoglin is detected in the absence of stimuli, it is probably carried out by a 'housekeeping' kinase. In this sense the inhibition of phosphorylation by H-7 suggests the involvement of PKC. Given the existence of a large family of PKC isoforms [22], the interaction of PKC with other signalling pathways and the possible cross-inhibition by H-7 of other Ser/Thr kinases, more detailed studies are required to identify the kinase/s responsible for endoglin phosphorylation.

Note added in proof (received 26 May 1994)

While this manuscript was under review, Yamashita et al. [23] reported that porcine endoglin is a phosphorylated protein which forms a heteromeric complex with signalling TGF- β receptors.

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