Kininogen binding protein p33/gC1qR is localized in the vesicular fraction of endothelial cells

Jürgen Dedio, Werner Müller-Esterl*

Institute for Physiological Chemistry and Pathobiochemistry, Johannes Gutenberg University at Mainz, D-55099 Mainz, Germany

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Abstract The endothelial protein p33/gC1qR is thought to mediate the assembly of components of the kinin-forming and complement-activating pathways on the surface of cardiovascular cells. FACS analysis of intact human umbilical vein endothelial cells using specific antibodies to p33 revealed a minor fluorescence on the cell surface whereas permeabilized cells showed a bright fluorescence indicative of an intracellular localization of p33. Immunostaining of fixed cells confirmed the predominant intracellular localization of p33. Fractionation studies demonstrated that the vesicular but not the membrane fraction of EA.hy926 cells is rich in p33. We conclude that externalization of p33 must precede its complex formation with target proteins on the endothelial cell surface.

Key words: Kininogen binding protein; p33; gC1qR; Kinin; Complement

1. Introduction

The assembly of hormone precursors on the surface of their target cells serves to secure the rapid and efficient release of the corresponding peptide hormones at or next to their site of action. This principle is exemplified by the kinin and the complement systems which are systemically available but locally acting. To facilitate the circumscribed release of effectors such as bradykinin and C5a in situ, target cells expose docking proteins that bind the interacting components of these systems and assemble them on cellular surfaces. The nature and function of such docking proteins has long been obscure. Recently we and others have identified a major kininogen binding protein, p33 of endothelial cells [1,2], that binds to H-kininogen with high affinity (9 nM) and anchors preformed H-kininogen-prokallikrein complexes to endothelial cell surfaces [3,4]. The same protein, gC1qR, serves as a 'receptor' for the globular heads of the complement component C1q and has been claimed to assemble the early complement factors on the surface of lymphocytes and neutrophils [5,6]. This latter view has been challenged by the recent observation of an intracellular localization of gC1qR in Raji and mesangial cells [7]. We wondered whether this phenomenon also extends to endothelial cells, the key players in the kinin-forming pathway of the cardiovascular system. Here we demonstrate that p33/gC1qR is almost exclusively localized in intracellular vesicles of endothelial cells, and conclude that externalization must precede interaction of p33/gC1qR with kininogen on the endothelial cell surface.

*Corresponding author. Fax: (49) (6131) 395792.
E-mail: werner.muller-esterl@uni-mainz.de

2. Materials and methods

2.1. Production and immunoselection of antibodies

Antisera to synthetic peptides or to recombinant p33 were raised in rabbit [8]. Peptide CHT28 of 28 residues derived from the NH_2-terminal portion of mature p33 was synthesized by the solid phase method. Recombinant p33 was expressed in *E. coli* in the form of a fusion protein with bacterial maltose binding protein (MBP), and purified as described [1]. The resultant antisera were tested with an indirect ELISA using free peptides (2 μg/ml) or recombinant p33 (0.5 μg/ml) as the coating antigen. For immuno-affinity chromatography peptide CHT28 (4 mg/ml) or recombinant p33 (2 mg/ml) were covalently coupled to Affi-Gel 10.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were prepared according to Jaffe et al. [9]. EA.hy926 cells [10] were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 10% (v/v) fetal bovine serum, 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, and antibiotics in a humidified 5% CO_2 atmosphere at 37°C.

2.3. Fluorescence-activated cell sorting (FACS)

Confluent HUVEC or EA.hy926 cells were removed from dishes with phosphate-buffered saline (PBS), pH 7.4, 0.5 mM EDTA, and washed 2× with ice-cold PBS, 0.5% (w/v) bovine serum albumin ('incubation buffer'). Cells (1×10^6) were suspended in incubation buffer containing antiserum at 1:100 (v/v) for 1 h at 4°C, washed 3×, and resuspended in incubation buffer containing fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig at 1:100 (v/v). Cells were incubated (1 h, 4°C), washed 3×, fixed with 4% (v/v) formaldehyde, and analyzed by FACScan (Becton Dickinson) and the Lysis II program. Alternatively the cells were fixed with 4% formaldehyde prior to antibody exposure. For permeabilization fixed cells were washed 2× with PBS and incubated with 0.1% Triton X-100 in PBS (5 min, 25°C).

2.4. Immunofluorescence studies of HUVEC and EA.hy926 cells

HUVEC or EA.hy926 cells were grown on chamber slides (Nunc). The cells were washed 2× with ice-cold PBS and fixed with 4% formaldehyde. For membrane permeabilization the cells were treated with ice-cold methanol for 10 min. After washing 3× with PBS, the cells were incubated (30 min, 37°C) with 20 μg/ml antibody in PBS, 0.5% bovine serum albumin. The cells were washed and incubated (30 min, 37°C) with FITC-conjugated goat anti-rabbit Ig at 1:100 (v/v) in the same buffer. After 3 washes with PBS, the cells were embedded in *n*-propyl gallate and viewed with an Axiophot microscope (Zeiss).

2.5. Cell fractionation

EA.hy926 cells were grown to confluence on 24 cm dishes, washed twice with ice-cold PBS, and scraped from the plates in 50 mM phosphate, pH 7.4, 0.28 M sucrose, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine·HCl. The cells were homogenized (Ultra-Turrax, Janke and Kunkel, Germany) with 2 strokes of 15 s each. The homogenate was centrifuged ($800 \times g$, 10 min), the nuclear pellet P_1 was discarded, and the supernatant S_1 was centrifuged again ($30\,000 \times g$, 20 min). Supernatant S_2 was collected, the membrane pellet P_2 was washed with 50 mM phosphate, pH 7.4, and resuspended in the same buffer containing 12% (w/v) sucrose. An aliquot (1 ml) was layered on top of a 33% sucrose cushion (11 ml) and centrifuged ($100\,000 \times g$, 3 h). The pellet P_3 (vesicular fraction) was resuspended in phosphate buffer. The membranes at the gradient interface were collected, diluted with 50 mM phosphate, pH 7.4 and

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centrifuged (30000×g, 45 min) [11]. Pellet P_4 (membrane fraction) was resuspended in phosphate buffer. Centrifugation (100000×g, 3 h) of supernatant S_2 gave pellet P_5 (microsomal fraction) and supernatant S_3 (cytosolic fraction). All manipulations were done at 4°C, and the cellular fractions were stored at −80°C until use.

2.6. Gel electrophoresis and immunoblotting

Harvested cells or cellular fractions were dissolved in sample buffer (63 mM Tris-HCl, pH 6.8, 2.5% SDS, 5% glycerol, 5% 2-mercaptoethanol, 0.005% bromophenol blue) for 30 min at 42°C, followed by SDS-PAGE on linear 12% (w/v) polyacrylamide gels. The separated proteins were electrotransferred to nitrocellulose membranes according to the manufacturer's instructions (Schleicher and Schuell). Immunoprints were done with 0.5–1 μg/ml of purified antibody applying the chemiluminescence method with a horseradish peroxidase-labeled secondary antibody at 1:5000.

2.7. Immunoprecipitation of ^{35}S-labeled p33

Confluent EA.hy926 cells were washed with Cys/Met-free DMEM, and incubated with the same medium (1 h, 37°C). The cells were labeled with 100 μCi/ml [^{35}S]Cys/Met (6 h, 37°C). The cell culture medium was harvested, adjusted to 10 mM EDTA, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine·HCl, and centrifuged (14000×g, 10 min). The supernatant was transferred to a fresh tube and incubated under rotation (1 h, 4°C) with 10 μl anti-p33 bound to *Staphylococcus A* cells (Calbiochem). The mixture was centrifuged (8000×g, 2 min, 4°C), and the precipitate washed 4× in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% deoxycholic acid, 1% Nonidet P-40, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine·HCl ('RIPA'). The labeled cells were washed 2× with ice-cold PBS, lysed in RIPA buffer under rotation (1 h, 4°C), and centrifuged (14000×g, 10 min, 4°C). The supernatant was treated with anti-p33 as above. The precipitates were dissolved in sample buffer and run on SDS-PAGE. The fixed gel was incubated with 15% sodium salicylate (30 min, 25°C), dried (2 h, 60°C) and exposed to a Fuji X-ray film (12 h, −80°C).

3. Results

Antisera to recombinant p33 fused to MBP (anti-p33) or to the synthetic peptide CHT28 derived from the NH_2-terminus of mature p33 (anti-CHT28) were raised in rabbits. The specificity of the affinity-purified antibodies was assessed by immunoblotting using total cellular extracts of HUVEC, EA.hy926 cells, or from *E. coli* expressing the MBP-p33 fusion protein (Fig. 1). A single 33 kDa band of HUVEC or

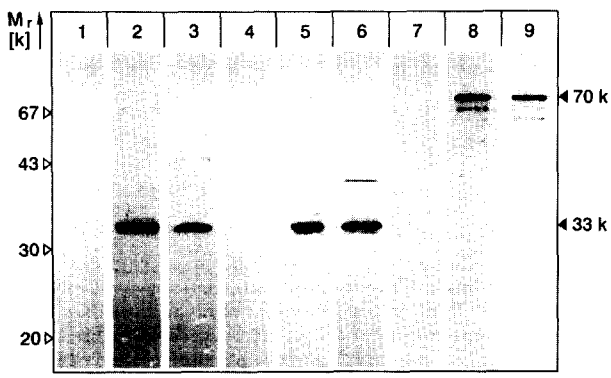

Fig. 1. Specificity of anti-p33 and anti-CHT28 demonstrated by Western blotting. Total cellular extracts (30 μg) of HUVEC (lanes 1–3) or EA.hy926 cells (lanes 4–6) were probed by preimmune sera at 1:1000 (lanes 1, 4), 0.5 μg/ml anti-p33 (lanes 2, 5), or 1 μg/ml anti-CHT28 (lanes 3, 6). For control, MBP-p33 present in total cellular extracts of *E. coli* was probed with preimmune serum (lane 7), anti-p33 (lane 8), or anti-CHT28 (lane 9). The molecular masses of standard (p33) proteins are indicated on the left (right).

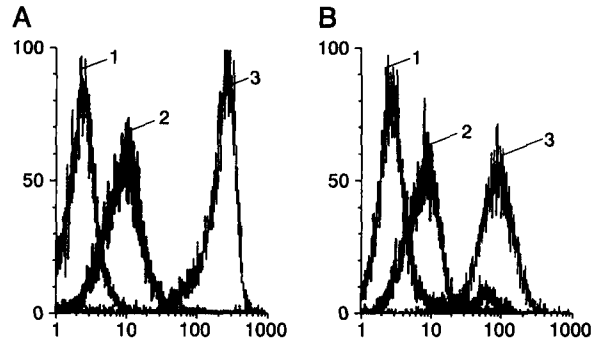

Fig. 2. FACS analysis of p33 expression on the surface of HUVEC (A) and EA.hy926 cells (B). Intact HUVEC and EA.hy926 cells were incubated with (1) preimmune serum, (2) anti-p33, or (3) permeabilized with 0.1% Triton X-100 and incubated then with anti-p33. The antiserum dilution was 1:100, and a secondary FITC-labeled antibody was used throughout.

EA.hy926 cell extracts was visualized by anti-p33 and by anti-CHT28 whereas the corresponding preimmune serum was negative (Fig. 1, lanes 1–6). In total cell extracts of *E. coli* a 70 kDa fusion protein of p33 and MBP was recognized by anti-p33 and anti-CHT28 but not by preimmune serum (Fig. 1, lanes 7–9). Hence anti-p33 and anti-CHT28 antibodies specifically and sensitively detect p33 in complex cellular extracts.

To test the cellular localization of p33, we performed FACS analyses of intact HUVEC (Fig. 2A) and EA.hy926 cells (Fig. 2B); preimmune serum served as the control. A minor increase in fluorescence intensity was seen with intact cells as compared to the control, whereas pretreatment of the cells with Triton X-100 markedly shifted the fluorescence signal to higher intensities. Similar results were obtained with anti-CHT28 (not shown). These data indicate that only a minor amount of p33 is accessible on the surface of endothelial cells whereas the vast majority of p33 is located within the cell borders.

To address the intracellular localization of p33, immunofluorescence studies were performed. Confluent monolayers of HUVEC or EA.hy926 cells were fixed, permeabilized, and stained with the anti-p33 (Fig. 3B–D) or preimmune serum (Fig. 3A). Both cell types showed a punctuate labeling juxtaposed to the nucleus whereas the cell surface was almost free of staining, suggesting that p33 is present in vesicles and not freely accessible in the cytoplasm.

The subcellular localization of p33 was further examined by fractionation of EA.hy926 cell homogenates. Equal amounts of protein from the various cell fractions were subjected to Western blotting using anti-p33 (Fig. 4A, upper panel). A 33 kDa band was found in the total cellular extract of EA.hy926 cells (lane 1), in the cytoplasm (lane 2), in the microsomal fraction (lane 3), and most prominently in their vesicular fraction (lane 5). By contrast the membrane fraction of EA.hy926 cells was almost free of p33 (lane 4). To verify the fractionation procedure we used a monoclonal antibody to a plasma membrane-associated enzyme, endothelial nitric oxide synthase (NOSIII, Affinity, Nottingham, UK). Unlike p33 the majority of NOSIII is present in the membrane fraction (Fig. 4A, lower panel), and the corresponding vesicular fraction is virtually free of the enzyme. These findings underline our notion that under the conditions of our experiments the majority of p33 is stored in intracellular vesicles whereas the

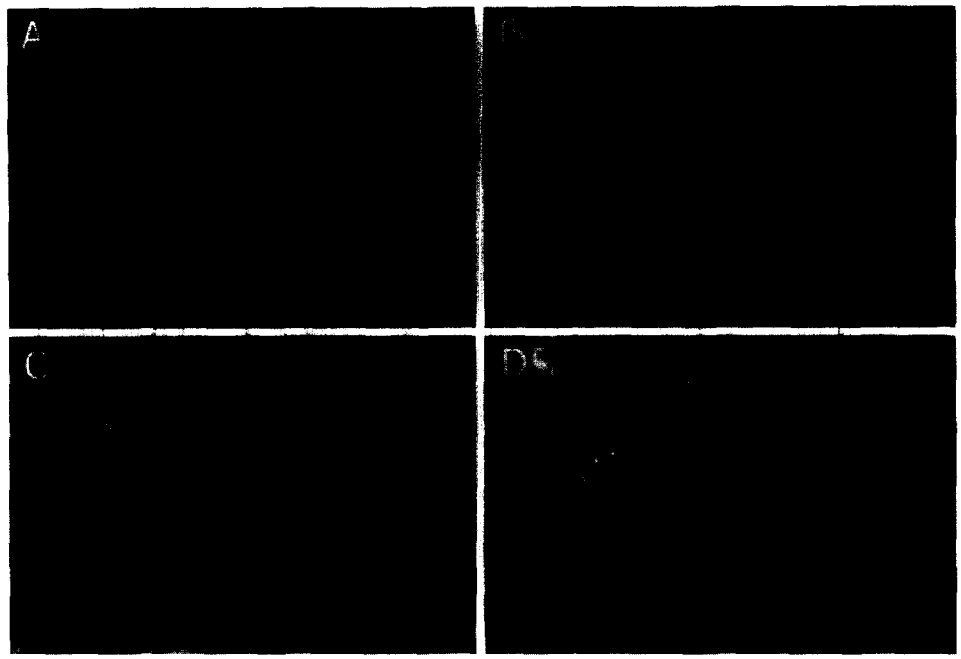

Fig. 3. Immunofluorescence of p33. EA.hy926 cells were incubated with preimmune serum at 1:100 (A) or with 20 μg/ml anti-p33 (B, C), and HUVEC with 20 μg/ml anti-p33 (D). Magnification ×40 (A, B), ×100 (C, D).

33 kDa band in the cytosolic fraction likely reflects lysis or mechanical disruption of vesicles during cell fractionation.

We asked if p33 is secreted from endothelial cells. To this end EA.hy926 cells were metabolically labeled with ^{35}S-amino acids, and immunoprecipitates were analyzed by SDS-PAGE and autoradiography (Fig. 4B). A major 33 kDa band and a minor 31 kDa band were immunoprecipitated from total cell lysates by anti-p33 (Fig. 4B, lane 2). These bands waned upon preincubation of anti-p33 with 100 μg MBP-p33 (lane 3), suggesting that the 31 kDa band represents a p33 degradation product of EA.hy926 cells. Preimmune serum failed to produce specific bands (lane 1). Unlike the cell lysate, the culture medium was virtually free of significant amounts of p33 (lane 4), indicating that EA.hy926 cells do not constitutively secrete significant amounts of newly synthesized p33 into the medium.

4 Discussion

In the present study, we have examined the localization of the kininogen binding protein, p33/gC1qR, in primary cultures of HUVEC and in a hybrid cell line, EA.hy926. Three independent approaches using two distinct antibodies together lead to the conclusion that p33 is primarily localized in intracellular stores, and at best a minor part of it is exposed on the surface of these endothelial cells. This finding is unexpected because (i) p33/gC1qR has been shown by immunohistochemistry to be almost exclusively localized on the surface of B cells [5], neutrophils [6] and mast cells [12]; (ii) p33/gC1qR has the features of a secretory protein that is likely to be exported to the exterior of the cell [5]; and (iii) the demonstrated capacity of p33/gC1qR to interfere with kininogen binding to endothelial cell surfaces [1,2]. One possible explanation for the observed intracellular localization in endothelial cells is that under the conditions of cell culturing p33 is synthesized and transiently stored in vesicles from which it translocates to the plasma membrane by hitherto unknown stimuli. However, we have been unable to promote p33 translocation by bradykinin (data not shown) which increases the number of kininogen binding sites present on endothelial cells [13]. On the other hand, we cannot exclude the distinct possibility that p33 is a docking protein that is transiently exposed on the cell surface and rapidly internalized to import kininogen and possibly C1q into protected compartments of the endothelial cell. Indeed, van Iwaarden and coworkers have reported the internalization of surface-bound kininogen by

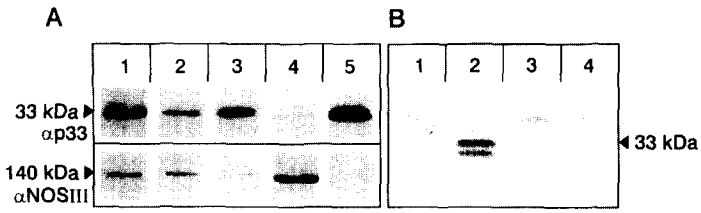

Fig. 4. A. Immunoprinting of p33 in subcellular fractions of EA.hy926 cells. Protein (20 μg per lane) from total cellular extract (lane 1), cytosolic fraction S_3 (lane 2), microsomal fraction P_5 (lane 3), membrane fraction P_4 (lane 4), or vesicular fraction P_3 (lane 5) were probed with 0.5 μg/ml anti-p33 (upper panel) or anti-NOSIII at 1:1000 (lower panel). B: Immunoprecipitation of p33. EA.hy926 cells were labeled with 100 μCi/ml of [^{35}S]Cys/Met. Total cellular extracts (lanes 1–3) or culture medium (lane 4) were probed with preimmune serum at 1:100 (lane 1), anti-p33 (lanes 2, 4), or anti-p33 preabsorbed with 100 μg/ml of MBP-p33 (lane 3). Immune complexes were recovered with *Staphylococcus A* cells. Molecular masses of the relevant proteins are indicated in the margins.

endothelial cells [14] but others have challenged this finding [15]. Our present efforts are directed towards the precise subcellular localization of p33. Identification of the cell compartment(s) containing p33 might provide us with new insights into the functional roles and modes of action of this versatile protein.

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