CD36 participates in the phagocytosis of rod outer segments by retinal pigment epithelium

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SUMMARY

Mechanisms of phagocytosis are complex and incompletely understood. The retinal pigment epithelium provides an ideal system to study the specific aspects of phagocytosis since an important function of this cell is the ingestion of packets of membranous discs that are normally discarded at the apical ends of rod and cone cells during outer segment renewal. Here we provide evidence that rod outer segment phagocytosis by retinal pigment epithelium is mediated by CD36, a transmembrane glycoprotein which has been previously characterized on hematopoietic cells as a receptor for apoptotic neutrophils and oxidized low density lipoprotein.

Immunocytochemical staining with monoclonal and polyclonal antibodies demonstrated CD36 expression by both human and rat retinal pigment epithelium in transverse cryostat sections of normal retina and in primary cultured cells. By western blot analysis of retinal pigment epithelial cell lysates, polyclonal and monoclonal antibodies to CD36 recognized an 88 kDa protein which comigrated with platelet CD36. Furthermore, the synthesis of CD36 mRNA by retinal pigment epithelium was confirmed by reverse transcriptase-PCR using specific CD36 oligonucleotides. The addition of CD36 antibodies to cultured retinal pigment epithelial cells reduced the binding and internalization of ^{125}I-labeled rod outer segments by 60%. Immunofluorescence confocal microscopy confirmed that outer segment uptake was significantly diminished by an antibody to CD36.

Moreover, we found that transfection of a human melanoma cell line with CD36 cDNA enabled these cells to bind and internalize isolated photoreceptor outer segments as seen by double immunofluorescent staining for surface bound and total cell-associated rod outer segments, and by measurement of cell-associated ^{125}I-labeled rod outer segments.

We conclude that the multifunctional scavenger receptor CD36 participates in the clearance of photoreceptor outer segments by retinal pigment epithelium and thus, participates in the visual process.

Key words: CD36, Rod outer segment, Retinal pigment epithelium

INTRODUCTION

Phagocytosis, the uptake by a cell of relatively large particles into vacuoles by clathrin-independent pathways, is of fundamental importance in tissue remodelling, wound healing, inflammation and host defences against pathogens (Greenberg and Silverstein, 1993). It is triggered by the interaction of specific molecules on the target particle surface with receptors on the phagocyte plasma membrane (Greenberg, 1995). The mechanisms by which cells recognize target molecules to trigger a phagocytic event are complex and vary among cell types. For instance, a large number of receptors have been identified that can act independently or in concert for a given cellular response. These have been placed into two functional families, opsonin-dependent (e.g. CR3 and FcR) (Brown, 1991; Ravetch, 1994) or opsonin-independent (e.g. mannose receptor, β_1 integrins and perhaps the type I/II scavenger receptor) (Ezekowitz et al., 1990; Isberg and Van, 1994; El Khoury et al., 1994). Why unique receptor complexes are required for phagocytosis of similar ligands remains unknown, as does the necessity for cooperative receptor utilization by a given cell.

Although the process of phagocytosis and the receptors involved have been extensively studied, the precise molecular mechanisms of receptor mediated phagocytosis remain relatively obscure because complex particles are often recognized by a number of different receptors, many of which remain incompletely characterized. The key elements among signals transduced from phagocytosis-promoting receptors that are necessary for specific changes leading to particle engulfment also remain to be identified (Greenberg and Silverstein, 1993). Part of the experimental conundrum in this field lays with the necessary study of primary cultures of phagocytes such as monocytes and macrophages, which although highly phagocytic and clinically significant, are difficult to obtain in large numbers, are difficult to work with in tissue culture systems and present complex phagocytic strategies (Rabinovitch, 1995). The retinal pigment epithelium (RPE), a monolayer of neuroecto-

dermally derived cells interposed between the light sensitive photoreceptor cells of the neural retina and the choroidal vasculature of the eye, provides an alternative system to study the specific mechanisms of phagocytosis (Bok, 1989). The RPE is responsible for phagocytosing packets of membranous discs which are shed from the apical ends of rod and cone outer segments into the subretinal space during the normal process of membrane renewal. The RPE possesses a phagocytic pathway that is specific and selective for rod and cone outer segment membranes (ROS) (Young and Bok, 1969). Each RPE cell participates in an enormous daily phagocytic flux of ROS through the apical membrane (Young, 1967). This phagocytic function is critical for normal vision as demonstrated by an animal model in which retinal degeneration and eventual blindness result from its failure (Bok and Hall, 1971; Edwards and Szamier, 1977). Experimentally, a pure RPE phagocytic system can be obtained using an established tissue culture model for ROS internalization, composed of primary cultured RPE and isolated ROS (Philp and Bernstein, 1981). Nevertheless, despite extensive experimental work (Colley et al., 1987; Shirakawa et al., 1987; Philp et al., 1988; Boyle et al., 1991; Gregory and Hall, 1992), the specific receptor-ligand system involved in ROS phagocytosis has not yet been conclusively identified (McLaughlin et al., 1994).

RPE cells are similar to macrophages not only in their ability to phagocytose, but also in the pattern of expression of certain surface receptors, including the mannose receptor and type I/II scavenger receptors (Shepherd et al., 1991; Hayes et al., 1989). Scavenger receptors are a receptor class that have been shown to recognize and mediate the internalization of chemically modified lipoproteins such as oxidized lipoprotein particles (OxLDL), acetylated LDL (AcLDL), polynucleotides and a wide variety of polyanions (Kreiger and Herz, 1994). At least three different classes of scavenger receptor have been identified, including receptors that recognize either AcLDL or OxLDL or both. The type I and type II scavenger receptors are homotrimers which are highly glycosylated and possess one transmembrane domain. Both of these receptors bind AcLDL and demonstrate specific polyanion binding (Kodama et al., 1990). A recently described family of scavenger receptors, including CD36, an 88 kDa integral plasma membrane glycoprotein, SRBI, a hamster homologue of CD36, and Cla-1 have been shown to bind oxLDL but not to have the broad polyanionic binding of the type I and II scavenger receptors (Nicholson et al., 1995; Acton et al., 1994).

CD36 was first identified and characterized on platelets and has since been found to be present on monocytes, macrophages, microvascular endothelial cells (Greenwalt et al., 1992), and adipocytes (Abumrad et al., 1993). CD36 was initially characterized as a receptor for the adhesive glycoproteins collagen and thrombospondin (TSP), and was shown to function in various cell-cell and cell-matrix adhesive interactions (Asch et al., 1987; Barnwell et al., 1989; Oquendo et al., 1989). Work from our laboratory and from others' suggests that CD36 accounts for greater than 50% of oxidized LDL uptake by macrophages, and thus may play an important role in the early pathogenesis of atherosclerosis (Nicholson et al., 1995; Acton et al., 1994).

Macrophage CD36 has also been demonstrated to play a role in inflammation by mediating the phagocytic clearance of senescent neutrophils (Savill et al., 1992; Ren et al., 1995).

TSP mediates this phagocytosis by acting as a 'molecular bridge' between the apoptotic neutrophil and both CD36 and the $\alpha_v\beta_3$ integrin on the macrophage surface. These two receptors appear to act together in their removal of apoptotic neutrophils from inflammatory sites, rather than through two separate, independent pathways (Ren et al., 1995).

The role of macrophage CD36 in the phagocytosis of lipoprotein particles and apoptotic neutrophils suggests that a parallel mechanism might be used in other non-macrophage phagocytic systems such as the RPE. In this paper, we report that RPE express abundant surface CD36 and that CD36 is involved in the recognition and internalization of ROS.

MATERIALS AND METHODS

Cell culture

Primary cultures of human RPE were established from human donor eyes (age 55-72 years old) obtained from the Eye Bank for Sight Restoration (New York, NY) within 12-18 hours of death. After excising the anterior segment, the vitreous and neural retina were removed by gentle suction and 0.2% trypsin (1:250; Difco Laboratories, Detroit, MI) was added to the posterior eye cup for 60 minutes at 37°C (pH 8.0). The trypsin was then replaced by Hepes-buffered Hanks' balanced salt solution (HHBSS) and the RPE cells were released from Bruch's membrane by agitation. The cells were collected in serum-containing HHBSS, centrifuged at 105 **g** for 4 minutes, resuspended in growth medium and plated at 200-500 cells/mm². Human RPE cultures were grown in DME (Gibco BRL, Gaithersburg, MD) with 20% heat inactivated FCS (Hyclone, Logan, UT), 2 mM glutamine (Gibco BRL), 0.1 mM MEM nonessential amino acid solution (Gibco BRL) and gentamycin sulfate (10 µg/ml; Schering Corp., Kenilworth, NJ) in 8-well (0.3 ml/well) plastic chamber slides (Lab-Tek; Nunc Inc., Naperville, IL). The cultures were maintained in a humidified environment at 37°C and 10% CO₂, with feeding twice weekly and were not passaged. The cells were grown for up to 6 weeks. To obtain human choroidal melanocytes, the cells were scraped from the inner surface of the sclera after the collection of RPE was completed. The melanocytes can be separated from capillary endothelial cells because the former are confined to the outermost layer of the choroid while the choriocapillaries lie within the inner choroid.

Bowes human melanoma cells (American Type Culture Collection, Rockville, MD) were maintained in DME supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicillin-streptomycin in a 5% CO₂ humidifed environment at 37°C. Bowes cell transfectants stably expressing CD36 have been previously described (Silverstein et al., 1992). Transfected cells were maintained in the same medium along with G418 (Gibco BRL) at 0.25 mg/ml.

Rod outer segment isolation

ROS were isolated by modification of the method of Molday et al. (1987). The isolation was performed under dim red light and sterile conditions using bovine eyes obtained from the slaughterhouse within 4 hours of death. The excised retinae were homogenized by gently shaking in a homogenization solution consisting of 20% sucrose, 65 mM NaCl, 1 mM MgCl₂, 10 mM glucose and 5 mM taurine in 20 mM Tris-HCl, pH 7.4. Subsequently, the large pieces of neural retina were pelleted by centrifugation at 1,000 rpm for 2-4 minutes and the suspension was filtered through sterile gauze to remove retinal fragments. The ROS containing supernatant was layered on the top of continuous sucrose gradients (27%-60%) and centrifugation was carried out in a Sorvall ultracentrifuge using a T865 rotor for 1 hour at 37,000 rpm and 4°C. The ROS fraction was identified as a faint orange colored band.

Proteins and antibodies

The murine mAbs to human CD36, 8A6 (Barnwell et al., 1989) and ESIVC7 were kindly provided by Dr John Barnwell (New York University, NY, NY) and Dr A. von dem Borne [University of Amsterdam via the Vth International Workshop on Leukocyte Differentiation Antigens (Silverstein et al., 1995)], respectively. Rabbit polyclonal antiserum to human CD36 was raised against purified, human platelet CD36 (Pearce et al., 1994). Rabbit polyclonal antiserum raised against rat CD36 was prepared by the subcutaneous inoculation of a New Zealand rabbit with 50 μg of CD36 protein (Abumrad et al., 1993) purified from the epidydimal fat pads of Wistar rats. Two boosters were injected 3 and 6 weeks after the initial inoculation with 50 μg of purified rat CD36 emulsified with Freund's incomplete adjuvant. Antisera was collected 2 weeks following each booster. IgG fractions were purified by affinity chromatography using a Protein A column (Pierce Biochemicals). Specificity of anti-CD36 IgG was confirmed by ELISA following the method of Harlow and Lane (1988). Rat CD36 was used as the antigen and alkaline phosphatase as the substrate.

Murine anti-TSP IgG, 11.4, was prepared as previously described (Silverstein and Nachman, 1987). Mouse mAb to rhodopsin (rho-4D2) was kindly provided by Dr Robert Molday (University of British Columbia, Vancouver, BC). Murine mAb to human $\alpha_v\beta_3$, 69-6-5, was obtained from Immunotech (Marseille, France) (Lehman et al., 1994). Human TSP and CD36 were purified as previously described (Sil-

verstein et al., 1989; Pearce et al., 1994). Murine RET-PE2 IgG (Neill and Barnstable, 1990) was a gift from Dr James Neill (Yale University, New Haven, CT). Rabbit anti-human von Willebrand factor antiserum was purchased from Dako Corporation (Carpinteria, CA). Rat monoclonal antibody to ZO-1 protein was purchased from the Development Studies Hybridoma Bank (University of Iowa, Iowa City, IA).

Immunohistochemistry

Eyes from Sprague-Dawley rats (juvenile) were fixed in 4% paraformaldehyde at 4°C for 8 hours, transferred to 30% sucrose and after embedding in OCT compound, 15 μM thick cryostat sections were prepared. The sections were blocked with 1% BSA and 5% normal goat serum with 0.1% Triton X-100 in PBS for 20 minutes and were treated with 50 mM NH_4Cl for 15 minutes. For double immunofluorescence labeling, the sections were incubated simultaneously with anti-CD36 (1:200) and RET-PE2 (1:50) for 60 minutes followed by washing and simultaneous incubation in FITC-conjugated goat anti-rabbit secondary antibody (1:50) and RITC-conjugated goat anti-mouse secondary antibody (1:50). Control sections were incubated in preimmune serum or in the absence of primary antibody. Adjacent sections were singly labeled with a rabbit polyclonal antibody to human von Willebrand factor (1:100) followed by FITC-conjugated goat anti-rabbit secondary antibody.
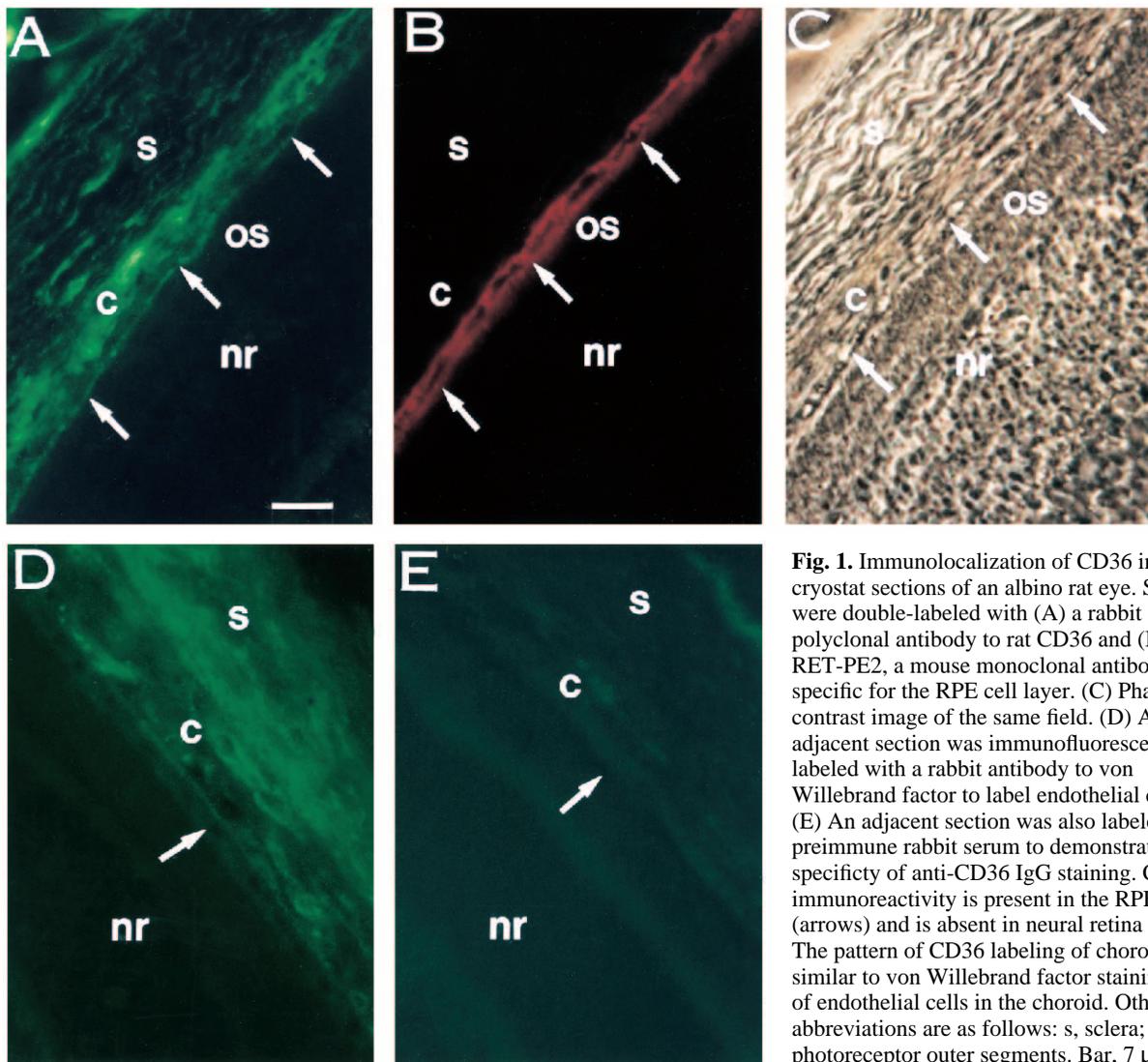
Fig. 1. Immunolocalization of CD36 in cryostat sections of an albino rat eye. Sections were double-labeled with (A) a rabbit polyclonal antibody to rat CD36 and (B) RET-PE2, a mouse monoclonal antibody specific for the RPE cell layer. (C) Phase contrast image of the same field. (D) An adjacent section was immunofluorescently labeled with a rabbit antibody to von Willebrand factor to label endothelial cells. (E) An adjacent section was also labeled with preimmune rabbit serum to demonstrate specificty of anti-CD36 IgG staining. CD36 immunoreactivity is present in the RPE (arrows) and is absent in neural retina (nr). The pattern of CD36 labeling of choroid (c) is similar to von Willebrand factor staining (D) of endothelial cells in the choroid. Other abbreviations are as follows: s, sclera; os, photoreceptor outer segments. Bar, 7 μM.

Western blotting

Cells were lysed in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 158 mM NaCl, 10 mM Tris, pH 7.2, 1 mM EGTA, 250 µg of leupeptin per ml, 0.1 mM phenylmethylsulfonyl fluoride, 1.0 mM Na_3VO_4 and 1 µM pepstatin) for 45 minutes at 4°C and separated by SDS-PAGE using 20 µg total protein per lane. Separated proteins were then transferred onto nitro-cellulose. After blocking for 60 minutes with 5% dry milk in TBS with 0.1% Tween-20 (TBS-T), the membrane was incubated in primary antibody (2 µg/ml, 60 minutes) in TBS-T, was washed in TBS-T (6× 5 minutes) and was incubated in secondary antibody conjugated to horseradish peroxidase (60 minutes). Immunoreactivity was detected by chemiluminescence with the ECL system (Amersham Corp., Arlington Heights, IL).

Reverse transcriptase PCR

Total RNA was isolated from both freshly harvested and cultured human RPE cells by cesium chloride gradients (Chirgwin et al., 1979). Single stranded cDNA was synthesized from RPE total RNA using Moloney Murine Leukemia virus reverse transcriptase and oligo $d(T)_n$ primers (Promega, Madison, WI). Negative controls included the following samples: no reverse transcriptase during cDNA synthesis, substitution of water for the cDNA template, no forward primer and no reverse primer. Amplification of the synthesized cDNA was performed with synthetic 24mer CD36 oligonucleotides. The forward primer began at nucleotide 156 and the reverse primer at nucleotide 601. Coordinates of the nucleotide pair correspond to the published human CD36 sequence (Oquendo et al., 1989) and the primer pair was designed to span intron-exon boundaries. DNA amplification was performed for 35 cycles.

Phagocytosis assays

Isolated ROS were diluted 3-fold in HHBSS, pelleted at 5,000 rpm for 20 minutes at 4°C and resuspended in growth medium with 2.5% sucrose (Mayerson and Hall, 1986) at a concentration of 5×10^7-1.5×10^8 ROS/ml. RPE cells were cultured in 8-well plastic Nunc chamber slides, overlaid with equal volumes of resuspended ROS and incubated for 2 hours in a humidified environment at 37°C and 10% CO_2. At the end of this incubation, the cells were gently rinsed free of unattached ROS using 10 ml of culture medium per well. Cells were fixed using 2% paraformaldehyde for 20 minutes at room temperature and were permeabilized and blocked with 0.1% Triton X-100 in 5% rabbit serum. Bound and ingested ROS were then detected with a mouse monoclonal antibody to rhodopsin, rho-4D2, and a FITC-conjugated rabbit anti-mouse Fab′2. The cell membranes were stained with a rat anti-ZO1 IgG and a Texas Red-conjugated rabbit anti-rat Fab′2. Cells were analyzed by confocal laser scanning fluorescence microscopy using a Sarastro 2000.

To quantify bound plus internalized ROS, isolated, intact ROS were radioiodinated according to the method of Clark and Hall (1982). Human or rat RPE cells, Bowes wild-type cells or Bowes CD36 trans-fected cells were cultured to confluency in 96-well plates. Preincubation of cells with CD36 mAb, control mouse IgG, anti-TSP mAb, anti-$\alpha_v\beta_3$ mAb or pretreatment of ^{125}I-ROS with soluble CD36 or soluble TSP was carried out in growth medium for 30 minutes at 37°C. Subsequently, equal concentrations of ^{125}I-ROS were added to the cells in the presence of the different treatments and the 96-well plates were centrifuged at 1,000 rpm for 4 minutes and incubated for 2 hours in a humidified environment at 37°C and 10% CO_2. Following this incubation, the cells were washed thoroughly with culture medium and treated with 100 µl of solubilization buffer (1%SDS, 0.5 M NaOH, 10 mM EDTA) for 30 minutes at 37°C. Cell-associated radioactivity was measured in a gamma counter and protein concentrations were determined by the BCA protein assay (Pierce Biochemicals). The data (dpm/µg protein) were analyzed by ANOVA to establish significant differences amongst treatment and control groups.

To count surface bound and total ROS, isolated ROS were fed to confluent cultures of human RPE cells, Bowes wild-type or Bowes CD36 transfected cells. Phagocytosis assays were performed as detailed above and staining of bound and total ROS was done following the specific method of Chaitin and Hall (1983) with the following changes: Rho-4D2, the anti-rhodopsin IgG was used to stain ROS while FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-mouse IgG were used as the secondary antibodies. Total ROS per cell were counted under flu-orescein filters while surface bound ROS were counted under rhodamine filters with 500-5,000 cells counted under both filters per experiment.

RESULTS

Expression of CD36 by retinal pigment epithelium

To determine whether the scavenger receptor CD36 is expressed by RPE, immunohistochemical staining of cryostat sections of normal rat retina was performed using a rabbit poly-clonal antibody raised against purified rat CD36 (Fig. 1A). To localize CD36 with respect to the RPE, tissue sections were immunostained concurrently for CD36 and an RPE specific marker, RET-PE2 (Fig. 1B). Strong immunoreactivity was observed in the RPE. Comparison of Figs 1A and B pho-tographed under fluorescein and rhodamine optics, respec-tively, illustrate that CD36 immunoreactivity co-localizes with RET-PE2 in the RPE monolayer thus demonstrating the expression of CD36 by RPE. CD36 immunoreactivity appears to be present as a a single line of fluorescence (Fig. 1A) while by comparison, RET-PE2 intensely labels both the basal and apical surfaces of the cells as well as the lateral membranes (Fig. 1B; Neill and Barnstable, 1990). We also observed CD36 immunopositive staining of the highly vascularized choroid, a finding which was expected since CD36 has been shown to be expressed by microvascular endothelium in many different tissues (Greenwalt et al., 1992). Indeed, an adjacent retinal section immunofluorescently labeled with rabbit anti-human von Willebrand factor antiserum labeled the endothelial cells present in the choroid (Fig. 1D). Specificity of staining by CD36 antiserum was demonstrated by the failure of preimmune rabbit serum to label the tissue sections (Fig. 1E). Primary cultures of both rat and human RPE also reacted with antibodies to CD36 as detected by immunocytochemistry (data not shown). Expression of CD36 by RPE was further confirmed by immunoblotting human RPE cell lysates (Fig. 2) as well as rat RPE cell lysates (data not shown). A polyclonal antibody raised against purified human platelet CD36 and mouse monoclonal antibodies to CD36 (ESIVC7 and 8A6) specifically recognized an 88 kDa protein in lysates from both cultured and freshly harvested human RPE. This protein comi-grated with an immunoreactive band corresponding to CD36 in platelet lysates. Conversely, neither lysates from freshly harvested human choroidal melanocytes nor lysates from freshly harvested neural retinal cells contained CD36. The absence of an immunoreactive band in the lysates of choroidal melanocytes excluded the possibility that the immunoreactiv-ity in the choroid was localized to melanocytes. Western blots probed with pre-immune serum or isotype matched control antibody failed to detect any bands in either the platelet or the RPE lysate.

To assess whether CD36 mRNA was present in RPE cells, reverse transcriptase (RT)-PCR analysis was performed using
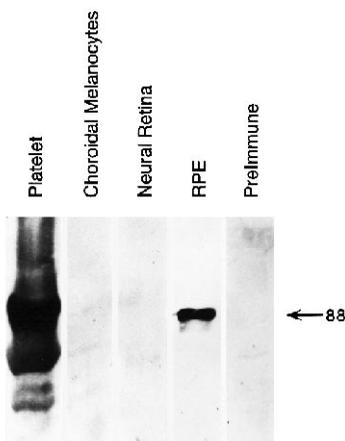
Fig. 2. Immunoblot analyses of CD36 expression in freshly harvested human RPE cells. Immunoblots were probed with murine anti-CD36 monoclonal antibody, ESIVC7 (2 µg/ml). The CD36 immunoreactive band in human RPE lysates comigrates with platelet CD36 while neither choroidal melanocyte cell lysates nor neural retinal cell lysates exhibit immunoreactivity. The preimmune labeled lane is an equivalent sample of human RPE lysates probed with an isotype matched control antibody with the absence of bands demonstrating the specificity of the anti-CD36 IgG probed blots. Molecular mass marker is given in kDa.

total RNA isolated from both freshly harvested or cultured human RPE cells. Using CD36 specific oligonucleotide primers, a 458 bp RT-PCR product was detected (Fig. 3). This product was identical in both size and restriction sites to the fragment amplified when CD36 plasmid was used as the template. However, this product was absent in samples lacking reverse transcriptase and in samples in which water was substituted for the cDNA template thus verifying the absence of contamination from either genomic DNA or any external sources.

CD36 participates in the binding and uptake of rod outer segment discs

To examine the role of CD36 in RPE phagocytosis of ROS, we used a quantitative in vitro phagocytosis assay with isolated ^{125}I-labeled ROS (Reid et al., 1992). Cultured human RPE incubated with isolated, radiolabeled ROS in the presence of
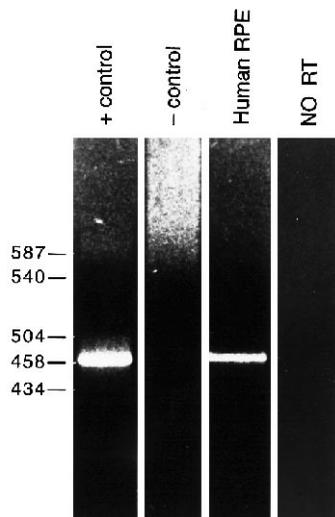

Fig. 3. RT-PCR detection of CD36 mRNA in cultured human RPE. For the positive control, a CD36 plasmid was used as the template in the PCR reaction and a 458 bp fragment was generated. For the negative control, water was substituted for the DNA template to control for contamination in the PCR reactions. A 458 bp fragment was also amplified from RNA isolated from human RPE cells. The NO RT lane is a sample in which no reverse transcriptase was added during cDNA synthesis to ensure the lack of genomic DNA contamination. Numbers in the margin denote apparent size in bases.

5-10× saturating concentrations of CD36 antibody, bound and took up significantly less radioactivity than cells incubated with control antibodies (Fig. 4). Inhibition by anti-CD36 IgG was 62.4% (±1.9% s.e.m., n=8; P<0.5) in comparison to untreated controls or isotype matched mouse IgG (with radioactivity exceeding that of untreated cells by 4.5%±2.2% s.e.m.; n=4). In order to ensure that antibody cross linking was not contributing to the inhibition of ^{125}I-ROS uptake by anti-CD36 IgG, the experiment was repeated using Fab fragments generated from anti-CD36 IgG and demonstrated similar levels of inhibition (data not shown). Incubation of RPE with an anti-$\alpha_v\beta_3$ integrin antibody did not result in a significant difference in the binding and uptake of ROS, as compared to untreated controls (inhibition being 12.2%±2.8% s.e.m., n=4; P>0.1). Using confocal microscopy, we also found that blocking CD36 inhibited the internalization of ROS by both human and rat RPE. Fig. 5 demonstrates images of normal adult rat RPE double immunolabeled with a monoclonal antibody to ZO-1, a tight junction protein localized to the RPE membrane, and with a monoclonal antibody to rhodopsin (rho-4D2), the visual pigment protein specific to rod photoreceptor cells, labeling the ROS. Staining with anti-ZO-1 IgG followed by a Texas Red-conjugated goat anti-rat IgG was performed to detect the borders of the cells and thus demonstrate the presence of immunostained ROS within and not between cells. To verify internalized ROS, a series of horizontal (x,y) sections was taken by confocal imaging beginning at the cell surface and moving vertically through the cells. Representative horizontal scans at depths of approximately 6 µM demonstrate that significantly fewer internalized ROS were found in the presence of an anti-CD36 antibody (Fig. 5A), in comparison to the presence of preimmune rabbit serum (Fig. 5B). The ROS appear yellowish because the green, FITC-staining ROS overlapped with the background levels of red staining cytoplasm. Similarly, human RPE challenged with ROS in the presence of anti-CD36 monoclonal antibody, 8A6 and stained by the double immunofluorescent method of Chaitin and Hall (1983), exhibited 64.9% less total ROS per field of 10 cells and 58.1% less surface bound ROS per field than RPE cells challenged in the presence of control IgG (Fig. 6). To ensure the specificity of CD36 in the inhibition of ROS binding and uptake, we examined the effect of CD36 antibodies on RPE phagocytosis of FITC-conjugated latex beads. As assessed by confocal microscopy, anti-CD36 IgG had no effect on the non-specific binding and ingestion of FITC-conjugated latex beads (data not shown) suggesting that the binding and uptake by CD36 is a specific receptor-mediated event.

To characterize further the role of CD36 in ROS phagocytosis, we investigated whether expression of CD36 by a normally non-phagocytic cell rendered it able to phagocytose ROS. A neuroectodermally derived human cell line (Bowes melanoma cells) was stably transfected with CD36 cDNA and shown to express abundant surface CD36 (Silverstein et al., 1992). These cells bound and internalized isolated ROS (Fig. 7A) as seen by epifluoresence microscopy and staining with an anti-rhodopsin monoclonal antibody, rho-4D2. In contrast, few immunoreactive particles were seen in association with control, non-transfected Bowes cells (Fig. 7B), or cells transfected with vector alone (data not shown). To quantitate ROS binding and uptake by CD36 transfected Bowes cells, we used the ^{125}I-ROS phagocytosis assay. Following incubation with
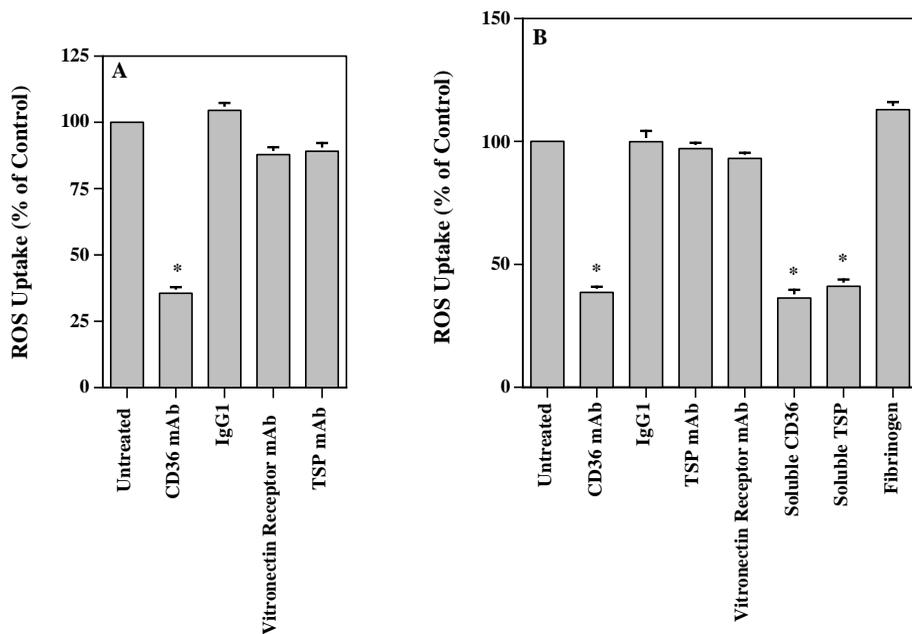
Fig. 4. Binding and uptake of ^{125}I-rod outer segments (ROS) by cultured human RPE cells and CD36-transfected Bowes cells. (A) ^{125}I-ROS binding and uptake by human RPE in the presence of the following antibodies at 10 µg/ml: anti-CD36 mAb (8A6), non-immune mouse IgG_1, anti-vitronectin receptor ($\alpha_v\beta_3$ integrin) mAb, and murine anti-TSP IgG (11.4). Results are presented as a percentage of ROS uptake by RPE alone (untreated control). (B) ^{125}I-ROS uptake by CD36-transfected Bowes cells in the presence of an anti-CD36 mAb (8A6), non-immune mouse IgG_1, murine anti-TSP IgG (11.4), anti-vitronectin receptor ($\alpha_v\beta_3$ integrin) mAb, or following pre-incubation of ROS with purified platelet CD36 (10 µg/ml), soluble TSP (10 µg/ml), or fibrinogen (10 µg/ml) is presented as a percentage of ROS uptake by CD36-transfected Bowes alone (untreated control). Note that the untreated control values are presented as 100% uptake. Values are mean ± s.e.m. of 4-9 independent experiments with each treatment performed in triplicate. *$P<0.5$.

^{125}I-ROS, the CD36-transfected Bowes cells demonstrated a 9-fold greater cell-associated radioactivity than did the wild-type cells. To show that this difference was due specifically to CD36 expression, we demonstrated that anti-CD36 monoclonal antibodies blocked binding and uptake by 61.2% (±1.7%, s.e.m.; n=6, $P<0.5$) (Fig. 4B). No effect was observed on Bowes wild-type cells (data not shown). Isotype matched nonimmune IgG (inhibition: 0.1%±4.4%, s.e.m., n=6; $P>0.1$) as well as anti-$\alpha_v\beta_3$ integrin IgG (6.9% inhibition ±2.3%, s.e.m., n=5; $P>0.1$) had no effect on the binding and uptake of ROS to transfected Bowes cells and was not significant in comparison to untreated controls. Pre-incubation of ^{125}I-ROS with purified platelet CD36 also diminished binding and uptake by CD36-transfected Bowes cells by 63.6% (±3.3% s.e.m.; n=4, $P<0.5$) while albumin had no effect (data not shown).

To determine the difference in ability of Bowes CD36 cells and Bowes wild-type cells to bind or internalize ROS, the double immunofluorescent staining method of Chaitin and Hall (1983) was performed. Bowes CD36 cells were found to have an average of 10.8 (±2.3 s.e.m.; n=8) internalized ROS per cell and 3.9 (±1.1 s.e.m.; n=8) surface bound ROS per cell. Meanwhile, Bowes wild-type cells were found to have an average of 0.8 (±0.9. s.e.m.; n=3) ingested ROS per cell and 2.6 (±1.3 s.e.m.; n=3) surface bound ROS per cell (Fig. 8).

Since thrombospondin (TSP) at the macrophage surface has been shown to participate in CD36-dependent phagocytosis of apoptotic neutrophils (Savill et al., 1992; Ren et al., 1995), we investigated the potential role of TSP in ROS phagocytosis by RPE and CD36 transfected Bowes cells. Using the in vitro phagocytosis assay, we found that the addition of soluble, purified TSP resulted in a 58.9% (±2.7% s.e.m.; n=5, $P<0.5$) decrease of ROS uptake by CD36 transfected Bowes cells (Fig.
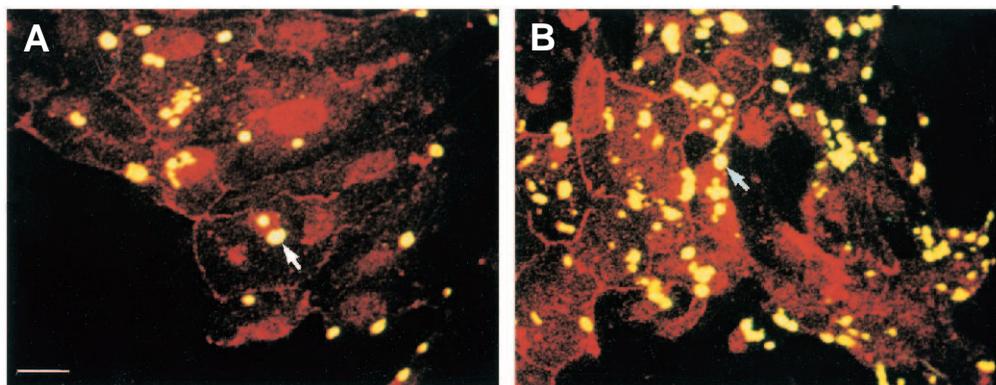

Fig. 5. Immunofluorescent confocal images of internalized rod outer segments (ROS) by adult Long Evans rat RPE cells in the presence or absence of CD36 antiserum. (A) RPE pre-treated with rabbit anti-rat CD36 IgG, then phagocytically challenged with ROS (arrow) in the presence of anti-CD36 IgG exhibited fewer internalized ROS than are seen in (B) a corresponding field of RPE similarly treated with preimmune control antiserum. These representative scans are approximately 6 µM beneath the cell surface with ROS detected by an anti-rhodopsin IgG, rho-4D2, followed by a FITC-conjugated rabbit anti-mouse IgG. RPE cell membranes are labeled with anti-ZO-1 IgG and a Texas Red-conjugated goat anti-rat IgG. The yellow fluorescence corresponds to the overlap of green and red. Bar, 15 µM.
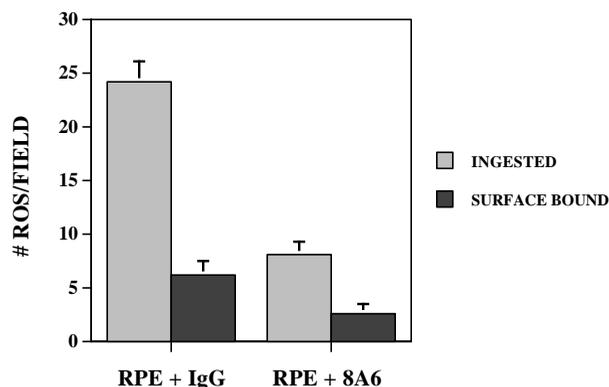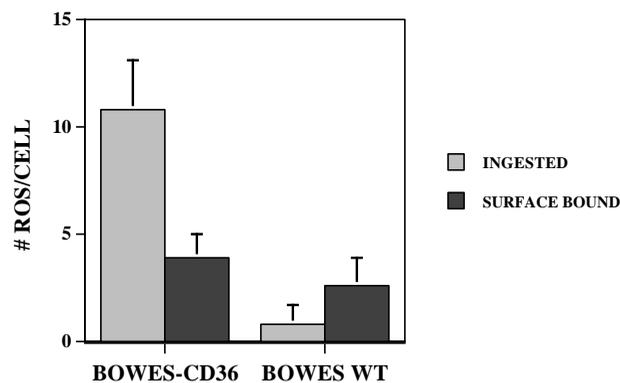
Fig. 6. Binding and internalization of ROS by human RPE in the presence or absence of anti-CD36 mAb. RPE were preincubated with anti-CD36 IgG (8A6) or control IgG then phagocytically challenged with ROS in the presence of 8A6 (10 µg/ml) or control IgG (10 µg/ml). ROS were immunostained with different fluorochromes before and after RPE cell membrane permeabilization to determine total and surface bound ROS per field of 10 cells with the number of ingested ROS calculated by subtraction. Values are mean ± s.e.m. of 8 independent experiments.

5B). The addition of albumin or fibrinogen, another large, extracellular matrix adhesion protein, had no effect on levels of ROS ingestion demonstrating specificity. That soluble purified TSP, another known ligand for CD36 (Asch et al., 1987), competes with ROS for binding to CD36 supports the experiments in which CD36 protein and CD36 antibodies block binding of ROS to CD36 on RPE and CD36 transfected Bowes cells. However, since TSP antibodies previously shown to block TSP-CD36 binding (Silverstein et al., 1989) did not block ROS uptake by either RPE cells (10.9% decrease ± 3.1% s.e.m., $n=4$; $P>0.1$) or by CD36 transfected Bowes cells (2.9% decrease, ±2.5% s.e.m., $n=4$; $P>0.1$), TSP is unlikely to be
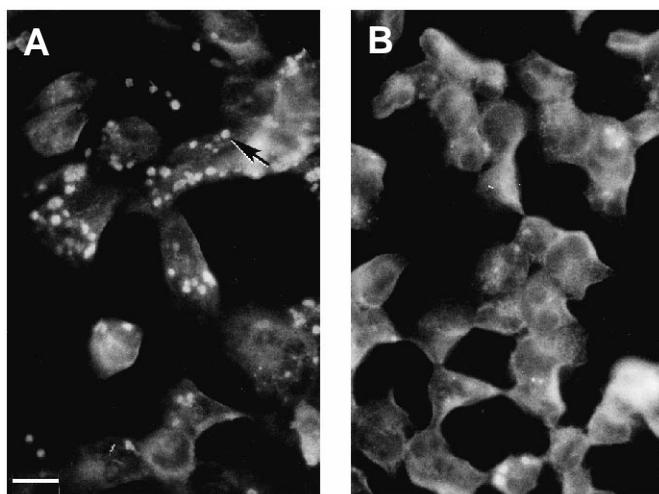
Fig. 7. Epifluorescent detection of bound and internalized ROS by CD36-transfected and wild-type Bowes cells following phagocytic challenge and immunolabeling with anti-rhodopsin mAb, rho-4D2. (A) CD36-transfected Bowes cells exhibit many rho-4D2 positive particles (arrow). (B) Bowes wild-type cells are not associated with rho-4D2 positive particles after phagocytic challenge. Bar, 14 µM.

Fig. 8. Binding and internalization of ROS by CD36 transfected and wild-type Bowes cells. Cells were challenged with ROS then double immunostained to count total and surface bound ROS with numbers of ingested ROS calculated by subtraction. Values are mean ± s.e.m. of 3-6 independent experiments.

involved in the binding and uptake of ROS in vivo as it is in senescent neutrophil uptake. Furthermore, immunohistochemical staining of human retinal sections, human RPE cultures, and isolated, purified ROS using monoclonal and polyclonal anti-TSP antibodies did not reveal any TSP expression (data not shown).

DISCUSSION

The present work provides converging evidence that the scavenger receptor CD36 participates in the binding and internalization of rod outer segments by the neuroepithelial-derived RPE. Rat and human RPE were shown to express CD36, and binding and uptake of ROS by RPE was inhibited by CD36 antibodies and soluble CD36 in vitro. In addition, a neuroepithelial-derived cell line which is non-phagocytic for ROS acquired the ability to bind and internalize ROS following transfection with CD36 cDNA.

The basic cellular process of receptor mediated endocytosis has been defined by examining the interaction between cells and various molecules, such as LDL. Receptor mediated endocytosis of a variety of modified particles which include LDL and albumin, has been collectively termed 'scavenger function' (Krieger and Herz, 1994). This physiological function is necessary for the removal of molecules modified by processes such as glycation or oxidation. CD36 has been identified as a member of a novel family of scavenger receptors, separate from the type I/II receptors, because of its recently discovered role in the binding and uptake of oxidized LDL. Its identification here as a specific receptor expressed by the RPE and its involvement in mediating the phagocytosis of ROS gives support to its functional classification as a scavenger receptor and confirms the broad physiologic significance of the scavenger receptor family.

Anti-CD36 antibodies have been found to inhibit approximately 50% of specific binding of oxLDL to CD36 in a macrophage-like cell line and on primary cultured monocyte-derived macrophages (Endemann et al., 1993; Nicholson et al., 1995). Similarly, ROS uptake by RPE cells upon inhibition of CD36 was also incompletely blocked, but in comparison to

oxLDL inhibition, the extent of inhibition was greater (60%). This incomplete inhibition may suggest the involvement of another protein or 'co-receptor' in ROS uptake. Indeed, our data does not preclude the involvement of a two receptor system mediating ROS phagocytosis with CD36 mediating internalization while the co-receptor recognizes and binds ROS, or vice versa. Precedence for this model is provided by the observation that CD36 acts in cooperation with the $\alpha_v\beta_3$ integrin to mediate uptake of apoptotic neutrophils. Blocking CD36 with antibodies resulted in a 40% inhibition of senescent neutrophil uptake while soluble CD36 inhibited uptake by approximately 20% inhibition. However, blocking both CD36 and the $\alpha_v\beta_3$ integrin inhibited uptake by 80% (Savill et al., 1992; Ren et al., 1995). We found in our system that blocking the $\alpha_v\beta_3$ integrin receptor with an inhibitory monoclonal antibody specific for this receptor and for the $\alpha_v\beta_5$ integrin, had no effect on ROS uptake by either RPE or CD36 transfected Bowes cells. The mannose receptor has been suggested to play a role in ROS phagocytosis by RPE (Boyle et al., 1991) and is another possible candidate co-receptor that may act with CD36 in mediating ROS internalization.

The ligand on the outer segment membranes recognized by CD36 is unknown, although several ligands for CD36 have been identified in other systems and are therefore candidates. As previously mentioned, TSP participates in the uptake of senescent neutrophils by mediating the interaction between the neutrophil and both the $\alpha_v\beta_3$ integrin and CD36 receptors at the macrophage surface (Savill et al., 1992). However, since TSP antibodies did not inhibit uptake and since we were unable to immunolabel ROS membranes or RPE using antibodies to TSP, it is unlikely that TSP participates in the binding and uptake of ROS in vivo. Since CD36 is also known to bind modified lipoproteins (Endemann et al., 1993; Nicholson et al., 1995), and may mediate fatty acid binding and/or transport (Abumrad et al., 1993; Jochen and Hays, 1993), modified lipids on the ROS membranes may serve as ligands for CD36. ROS membranes are known to be rich in long chain polyunsaturated fatty acids (Wang and Anderson, 1993) and are highly susceptible to oxidation in the subretinal space (Wiegand et al., 1983; Zimmerman and Keys, 1991). It is reasonable to speculate, therefore, that before or during the shedding process, the ROS membranes could become modified by peroxidation leading to exposure of a CD36 binding site. Nevertheless, immunostaining of isolated, fixed preparations of ROS did not reveal reactivity with either a monoclonal antibody against malondialdehyde-LDL or antibodies against negatively charged phospholipids (Gharavi et al., 1992; Wagenknecht and McIntyre, 1993) (unpublished observations).

CD36 has been found to associate with microinvaginations of the plasma membrane, known as caveolae. Since caveolae appear to function in endocytosis, ROS binding and internalization by CD36 may involve caveolae-mediated uptake. Endothelial cell caveolae have been found to bind, endocytose and transcytose LDL and modified forms of LDL (Lisanti et al., 1994), which may further support the possibility that the ligand for CD36 is a modified lipid on the ROS membrane.

The importance of identifying CD36 as necessary for ROS phagocytosis by RPE is twofold. Firstly, the role of CD36 as a scavenger receptor has been previously described only in relation to pathologic processes. Here we describe a significant function for CD36 in normal physiology. Secondly, we have identified CD36 on the RPE cell surface as a mediator of a critically important process: the phagocytosis of membranous discs shed from photoreceptor cell outer segments. These findings may have implications for the development of therapeutic strategies for some forms of retinal degenerative diseases.

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