Differential Roles of CD36 and αvβ5 Integrin in Photoreceptor Phagocytosis by the Retinal Pigment Epithelium

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Abstract

Retinal pigment epithelial (RPE) cells employ αvβ5 integrin and CD36 receptors to phagocytose photoreceptor outer segment fragments (OS). We explored special properties of RPE phagocytosis to identify the contribution of CD36 to RPE phagocytosis measuring effects of CD36 antibodies on OS binding and internalization kinetics. Early, CD36 antibodies had no effect on OS binding or internalization. Both control and CD36 antibody treated RPE initiated internalization approximately 2 hours after OS challenge. Later, bivalent CD36 IgG accelerated OS engulfment while monovalent Fab fragments inhibited engulfment. Cross-linking Fab fragments restored the accelerating activity of intact IgG. Strikingly, antibodies were effective even if added to OS already bound by RPE. αvβ5 blocking antibody reduced OS binding equally well in the presence of CD36 antibodies but CD36 antibodies accelerated internalization of remaining bound OS. Furthermore, CD36 ligation at either apical or basal RPE surface partially substituted for soluble factors that are required for internalization but not for binding of OS at the RPE apical surface. Our results demonstrate that CD36 ligation is necessary and sufficient to activate the OS internalization mechanism of RPE. They suggest that CD36 acts as a signaling molecule in postbinding steps of RPE phagocytosis independently of the OS binding receptor αvβ5 integrin.

Key words: phagocytosis • recognition • CD36 • integrins • retinal pigment epithelium

Introduction

Clearance phagocytosis of spent photoreceptor outer segment fragments (OS)* by the retinal pigment epithelium (RPE) is critical for the long-term maintenance of the retina (1, 2). In the retina, the apical surface of each RPE cell faces ~30 outer segments. Shedding of outer segment distal tips every morning, synchronized by light and circadian rhythms, precedes a burst of phagocytic activity by the RPE that efficiently removes shed OS from the retina (3). Since they do not normally turn over in the adult mammalian eye, each RPE cell phagocytoses shed OS once a day over many decades.

Exploring the phagocytic activity retained by RPE in culture, four plasma membrane receptors of the RPE have been described to participate in OS phagocytosis, a mannose receptor (4), a receptor tyrosine kinase known as Mer (5), αvβ5 integrin (6–8), and the type B scavenger receptor CD36 (9). αvβ5 integrin is the primary binding receptor used by human and rat RPE cells to bind OS (6). Interestingly, RPE cells also employ their αvβ5-dependent recognition mechanism to select and bind apoptotic cells (10). Irradiated thymocytes or aged neutrophils quantitatively compete with the RPE's native phagocytic particle, shed OS, for binding to αvβ5 in RPE and to αvβ3 in macrophages. This implies that αvβ3 and αvβ5 integrins perform similar functions in particle recognition by these two types of phagocytes.

The scavenger receptor CD36 may also perform equivalent tasks in clearance phagocytosis by RPE and by macrophages as its transfection into nonphagocytic Bowes melanoma cells renders them phagocytic toward apoptotic cells as well as toward OS (9, 11). Despite longstanding evidence that CD36 participates in clearance phagocytosis, the precise role of CD36 in the phagocytic mechanism of any cell type

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**Abbreviations used in this paper:* LDL, low-density lipoprotein; OS, photoreceptor outer segment fragments; oxLDL, oxidized LDL; RPE, retinal pigment epithelium.

remains unclear. CD36 can interact with phosphatidylserine (12, 13), a key "eat me" signal recognized by phagocytes (14), but a direct interaction of CD36 with phagocytic particles has not been shown. While earlier reports assigned a role for CD36 in cooperation with αvβ3 integrin in apoptotic cell clearance by monocyte-derived macrophages (15), recent data have shown that CD36 also participates in integrin-independent uptake pathways (16). Finally, previous studies did not precisely discriminate between putative roles of CD36 in particle recognition and in particle internalization.

The experiments in this report make use of special properties of the RPE's uptake mechanism to define the individual function of CD36 in OS clearance phagocytosis. Phagocytosis by RPE is a very slow process lasting ~2 h in the eye (3, 17) and even longer under culture conditions although it is independent of protein biosynthesis (18). Whether the delayed time course of OS uptake by RPE under experimental conditions reflects a less efficient uptake mechanism of RPE in culture as compared with RPE in vivo, or a lack of surface "eat me" signals by experimental OS preparations as compared with OS shed naturally, or a combination of both, has so far not been examined. Since OS binding by cultured RPE precedes particle internalization one can separate both events experimentally by choosing appropriate time points for analysis. Furthermore, binding is inhibited nearly completely by Abs to the OS binding receptor αvβ5 integrin (6). Internalization requires temperatures of at least 30°C as well as the presence of serum proteins, while binding proceeds at temperatures of 18°C and up regardless of soluble factors (18, 19). Heat inactivation does not alter serum activity ruling out a role for complement functions in the serum effect (19). To activate internalization of bound OS, serum must be present at the apical, phagocytic RPE surface indicating that RPE in culture carry apical serum factor receptors that may be part of the RPE phagocytic machinery (20). Miceli et al. showed that vitronectin increased OS uptake by adult human RPE in culture in the absence of serum but they did not measure OS binding (7). Since αvβ5 integrin Abs reversed the effect of vitronectin, vitronectin in their assays may have primarily affected OS binding.

We investigated the effects of CD36 Abs shown previously to affect CD36 functions including apoptotic cell phagocytosis on the kinetics of OS binding and internalization by RPE. Our results demonstrate that CD36 does not act during OS binding, the αvβ5 integrin-dependent phase of RPE phagocytosis. Furthermore, our experiments suggest that CD36 ligation by Abs or other ligands, including lipoproteins or unidentified serum factors, regulates the rate with which RPE cells internalize surface-bound OS. As CD36 ligation at the basal surface of RPE is equally effective as its ligation at the apical, phagocytic surface, CD36 likely functions primarily as a signaling molecule during RPE phagocytosis of OS.

Materials and Methods

Materials. Reagents were from Sigma-Aldrich or GIBCO BRL unless otherwise stated. FBS (total protein 37.5 mg/ml; ICN Biomedicals) and lipoprotein deficient FBS (total protein 35 mg/ml; Intracel) were heat inactivated for 30 min at 56°C. Human low-density lipoprotein (LDL) was obtained from Intracel and copper-oxidized using established procedures (21). Function blocking, heterodimer selective, rat and human αvβ5 reactive Ab clone P1F6 (22) was from Chemicon. Rat CD36 antiserum was described previously (9, 23). Purified IgG was used in function blocking experiments. For immunofluorescence of rat CD36 and coating of glass coverslips, we used an anti–murine CD36 mouse monoclonal IgA generated by immunizing CD36 null mice with recombinant adenovirus expressing full-length mouse CD36 cDNA. Flow cytometry and immunoprecipitation confirmed reactivity of the Ab with CD36 in wild-type mouse macrophages. No reactivity was observed with CD36 null mouse macrophages (unpublished data). For immunoblot detection of human CD36, we used rabbit antiserum raised against purified human platelet CD36 (24). For immunofluorescence labeling and function blocking of human CD36, Ab FA6–152 (25) was purchased from Immunotech. Secondary and nonimmune Abs were from Rockland or Jackson ImmunoResearch Laboratories.

Cell Culture. Human ARPE-19 cells (26) and rat RPE-J cells (27) (both from American Type Culture Collection) were maintained in DMEM supplemented with 10% or 4% FCS, respectively. For phagocytosis experiments, RPE-J cells were seeded and differentiated on matrigel (BD Biosciences) coated Transwell® semipermeable polycarbonate filters (Corning) as described previously (27). Alternatively, RPE-J cells were seeded at 50% confluence on glass coverslips and grown for 7–8 d. ARPE-19 cells were seeded at confluence on coverslips coated with matrigel 10–14 d before use.

Preparation of Photoreceptor OS. OS were isolated following established protocols from bovine eyes obtained fresh from the slaughterhouse (28) and stored in 10 mM Na-phosphate, pH 7.2, 0.1 M NaCl, 2.5% sucrose at −80°C.

Before use, OS were thawed and dyed by addition of 20% volume of 1 mg/ml FITC (Molecular Probes) in 0.1 M Na-bicarbonate, pH 9.0, for 1 h at room temperature in the dark, washed, and resuspended in cell culture medium. OS preparations were positive for immunolabeling with opsin Abs and negative for labeling with human or murine CD36 Abs used in this study. CD36 ligation had identical effects on fresh and thawed bovine OS, labeled with fluorescein or not, which competed with each other for binding and internalization by RPE (data not shown).

OS Binding and Phagocytosis Experiments. Unless otherwise stated, differentiated RPE monolayers were challenged with 10 OS per cell in DMEM with 3% FCS for the duration of the experiment, washed three times with PBS containing 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$ (PBS-CM) and fixed in ice-cold methanol. To quantify internalized OS only, FITC fluorescence derived from bound OS was quenched using Trypan's blue before fixation (6, 29). RPE nuclei were counterstained with propidium iodide at 1 ng/ml in PBS-CM. Samples were mounted on glass slides and evaluated by microscopy and fluorescence scanning.

Phagocytosis by RPE-J Cells Seeded on Coated Glass Coverslips. 5-mm glass coverslips in 96-well plates were incubated with CD36 IgA or control IgA at 10 μg/ml in PBS for 2 h at room temperature and with 2% FCS in DMEM for 1 h immediately before seeding RPE-J cells at a density of 2.5×10^5 cells/cm^2 in growth medium. Different coating Abs did not affect RPE-J viability, attachment, or spreading on glass coverslips. After 6 h, cells were fixed and stained or challenged with OS as described previously. Incubating fixed samples with FITC-conjugated IgA Abs confirmed that nonimmune and CD36 IgA-coated coverslips

evenly and persisted on the glass for the duration of the experiment.

Calculation of OS Binding and Phagocytosis Indices. Binding and internalization of OS was quantified by fluorescence scanning of samples as described in detail previously (6, 10) using a STORM 860 Imager (Molecular Dynamics). Areas of $\sim 2 \times 10^5$ RPE cells were selected and their fluorescence emission was quantified with ImageQuant 1.2 (Molecular Dynamics). To normalize OS counts for different cell densities, the fluorescence of propidium iodide (nuclei, red) and the OS-derived FITC fluorescence were both measured in each field. The binding index (determined by subtracting internalization counts from total OS counts) or the internalization index (measured directly after Trypan blue quenching of external OS) were calculated dividing OS fluorescence counts of each area by nuclei counts, thereby normalizing for RPE numbers. Microscopic observation revealed that ~80% of human or rat RPE cells phagocytosed multiple FITC-OS (on average 5) during 5 h of OS challenge. Using the double fluorescence scanning method, this translated into an OS internalization index of 6.21 ± 0.78 for ARPE-19 and 6.48 ± 0.61 for RPE-J cells (average ±SD).

Immunofluorescence Microscopy. Samples were fixed in ice-cold methanol or 4% paraformaldehyde in PBS-CM and processed as described previously (30). Samples were observed with a Nikon fluorescence microscope E600. Digital images were acquired with a back-illuminated cooled CCD camera (CCD1000 PB; Princeton Instruments), translated using MetaMorph (Universal Imaging) and recompiled in Photoshop v.5.0 (Adobe). Horizontal (x-y) sections were acquired at 0.5 μm steps using a z motor (Prior), and out of focus light was removed using MetaMorph.

Immunoblot Analysis. Cells were solubilized in 50 mM Tris/HCl, pH 7.8, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% SDS, 1% Na-desoxicholate, 1% Triton X-100, supplemented with 2 mM each of aprotinin, leupeptin, pepstatin, iodoacetamide and PMSF, and 1 mM N-ethylmaleimide by agitating for 30 min at 4°C. Protein concentrations in lysates were determined according to Bradford (31) and equal amounts of protein of each sample were separated on 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Blots were incubated with CD36 Abs and horseradish peroxidase conjugated secondary Abs followed by ECL detection (NEN Life Science Products). X-ray films were scanned and signals quantified using NIH Image 1.61.

Results

Stable RPE Cell Lines Express CD36. We previously established that CD36 participates in the phagocytic mechanism of rat primary RPE, and that CD36 transfection renders human melanoma cells phagocytic for OS (9). As with primary RPE, stable RPE cell lines derived from rat (RPE-J) or human (ARPE-19) RPE phagocytose OS and use αvβ5 integrin to recognize OS (6). To determine whether these RPE cell lines also serve as a model system to study the role of CD36 in OS phagocytosis, we assessed CD36 expression by immunoblotting and immunofluorescence microscopy. As shown in Fig. 1, both RPE-J and ARPE-19 cells expressed CD36; the amount of CD36 protein in RPE-J cells, as determined by immunoblot, was ~80% of that in adult rat RPE (Fig. 1 A). Immunofluorescence staining showed that CD36 localized to the plasma membrane of confluent ARPE-19 cells (Fig. 1 C). Parallel samples stained with nonimmune mouse IgG did not fluoresce (Fig. 1 D).

CD36 Abs Alter the Kinetics of OS Internalization by RPE but Do not Affect OS Binding. The mAb FA6–152 inhibits multiple CD36 functions including binding of modified LDL (32), inhibition of angiogenesis by thrombospondin-1 (33), and uptake of apoptotic cells (34). Therefore, we tested whether FA6–152 altered OS binding and/or internalization by ARPE-19 cells. We challenged the cells with FITC-OS for a period of 5 h in the continuous presence of control mouse IgG or FA6–152 within a range of concentrations up to 100 μg/ml. FA6–152 at concentrations of 20 to 50 μg/ml increased the amount of internalized OS present in RPE twofold over that of controls (Fig. 2). In contrast, at 75 and 100 μg/ml, the Ab reduced the amount of internalized OS by approximately one-third (Fig. 2). After 5 h of OS challenge, control cells had internalized 56% of their total FITC-OS, while RPE treated with 50 μg/ml FA6–152 internalized 79% and RPE treated with 100 μg/ml FA6–152 internalized only 26% of their total OS load. There were no statistically significant differences in the numbers of bound OS among the different treatments. The changes in internal OS drastically shifted the internal to ex-

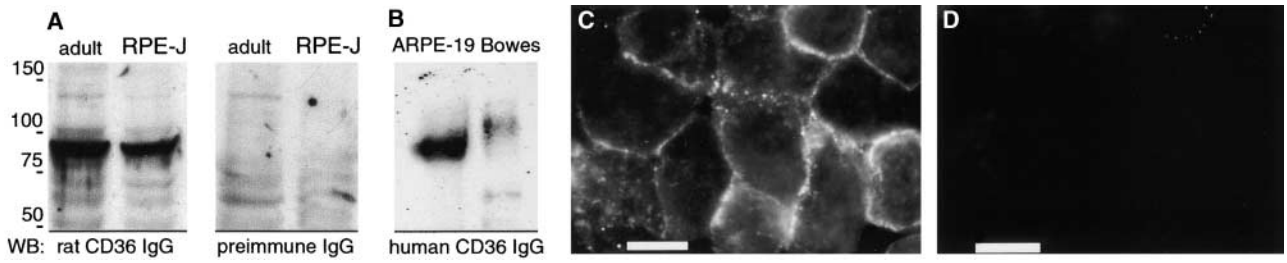


Figure 1. CD36 protein expression by stable human and rat-derived RPE derived cell lines. (A) Comparative immunoblotting of detergent extracts containing 20 μg total cellular protein followed by detection with rat CD36 antiserum shows that rat RPE-J cell lysates (RPE-J) contain ~80% of the CD36 protein detected in lysates of RPE prepared from adult rat eyes (adult). Bands are specific for CD36 immunization, since they are absent in Western blot analysis probed with preimmune IgG. (B) Comparative immunoblotting of detergent extracts containing 100 μg total cellular protein followed by detection with human CD36 antiserum reveals the presence of CD36 protein of the expected molecular size of ~88 kD in human ARPE-19 cells (ARPE-19) but not in human Bowes melanoma cells (Bowes), which are known not to express CD36 (reference 11). (C) Immunofluorescence labeling of paraformaldehyde fixed ARPE-19 cells with human CD36 mAb FA6–152 shows CD36 signals localizing to the RPE cell surface. (D) FA6–152 signals are specific since they are absent in parallel samples stained with nonimmune IgG.

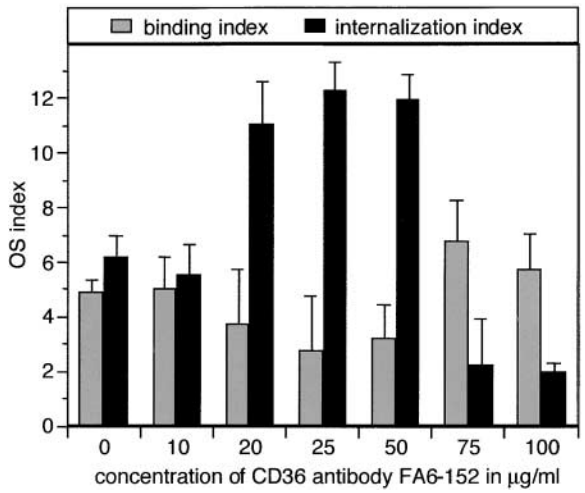


Figure 2. Concentration dependent effect of human CD36Ab FA6–152 on OS binding and internalization by ARPE-19 cells. Confluent, differentiated monolayers of ARPE-19 cells were challenged with FITC-labeled OS in the presence of 50 μg/ml nonimmune mouse IgG or increasing concentrations of FA6–152 as indicated for 5 h. Total (bound plus internalized) OS and internal (not quenched by Trypan's blue) OS were measured using fluorescence scanning and used to calculate the OS binding index (gray bars) and the OS internalization index (black bars) for each condition. The value of each bar represents the average OS index ± SD of four independent experiments. OS binding differences between samples were not statistically significant ($P = 0.1$). Student's t test analysis determined significant differences in OS internalization in the presence of 20–100 μg/ml FA6–152 as compared with control conditions ($P < 0.001$).

ternal OS distribution of RPE treated with different concentrations of FA6–152.

We next investigated the effect of FA6–152 on the kinetics of OS binding and internalization by ARPE-19 cells. Fig. 3 A shows that, at early time points up to ~2 h, during which RPE cells bind but do not internalize OS (6), all cells bound OS at the same rate regardless of Ab treatment. After 2 h, the time of onset of internalization by cultured RPE, cells treated with the inhibitory concentration of FA6–152 (100 μg/ml) had identical numbers of surface-bound OS as control cells. In contrast, cells treated with the stimulatory concentration of FA6–152 (50 μg/ml) had 15 to 35% fewer surface-bound OS than control cells. The decrease in cell surface-bound OS was probably due to an increased rate of internalization. Indeed, FA6–152 had opposite effects on the kinetics of OS internalization, confirming that the stimulating concentration of Ab accelerated engulfment beginning at 2 h while the higher concentration strongly inhibited engulfment (Fig. 3 B).

Similar results were seen with the rat RPE cell line RPE-J. Figs. 3 C and D show the effects of anti rat CD36 IgG at 50 μg/ml on the kinetics of OS binding (Fig. 3 C) and internalization (Fig. 3 D) of RPE-J cells. As with the human cells, CD36 IgG did not alter OS binding, but specifically increased the amount of internalized OS at all times points later than 2 h after OS addition. Similar results were obtained adding CD36 IgG to RPE-J cells at 20 μg/ml (data not shown).

Bivalency of CD36 Abs Is Required to Increase but not to Decrease the Rate of OS Internalization by RPE. The concentration dependence of their effects suggested that the Abs may act by cross-linking CD36 receptors at the RPE surface. Therefore, we tested the effects of monovalent Fab fragments of FA6–152 or of anti–rat CD36 IgG on OS internalization. Unlike the intact Ab, Fab fragments did not accelerate OS internalization (Fig. 4). However, addition of secondary Abs to cross-link rat CD36 Fab fragments par-

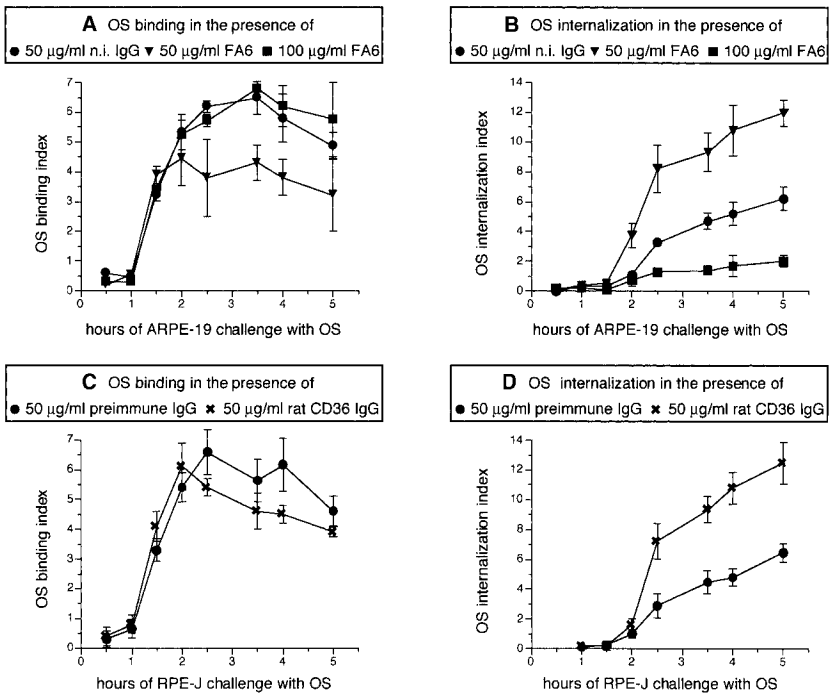


Figure 3. Effects of stimulating and inhibiting concentrations of CD36 Ab on the kinetics of OS binding and internalization by human ARPE-19 and rat RPE-J cells. Differentiated ARPE-19 cells were challenged for different periods of time up to 5 h with FITC-OS in the presence of 50 μg/ml nonimmune mouse IgG (●), 50 μg/ml FA6–152 (▼), and 100 μg/ml FA6–152 (■). For all time points, binding indices (A) and internalization indices (B) were determined as described for Fig. 2 and plotted against the time after OS challenge. Differentiated monolayers of rat RPE-J cells were challenged with FITC-OS as described for ARPE-19 cells in the presence of IgG fractions of preimmune serum (●) or rat CD36 antiserum (**x**), both at 50 μg/ml. Binding and internalization indices were determined as described for ARPE-19 cells and plotted against the time of OS challenge in (C) and (D), respectively. Values given are average OS index ± SD of three independent experiments for ARPE-19 cells and of five independent experiments for RPE-J cells. n.i., nonimmune.

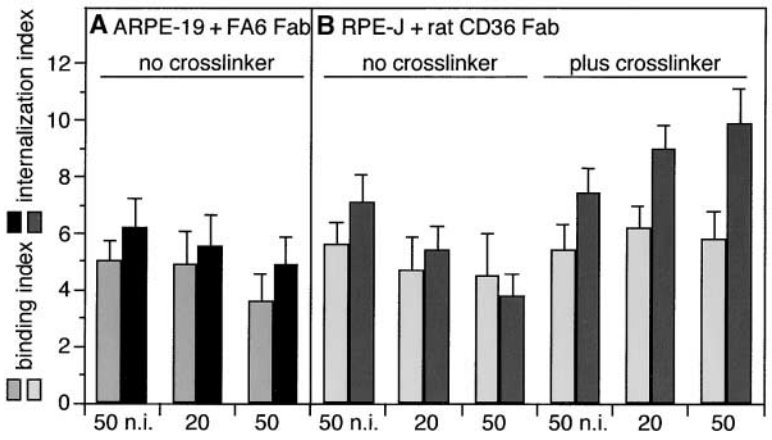


Figure 4. Inhibition but not stimulation of OS internalization by monovalent Fab fractions of CD36 Abs. 5 h of challenge with FITC-OS of human (A) and rat (B) RPE was followed by fluorescence scanning to calculate binding and internalization indices as described in Fig. 2. Light bars represent the binding index, dark bars the internalization index for each condition. Values in A and B represent averages ± SD of three independent experiments. (A) The presence of Fab fractions of FA6–152 IgG at 20 and 50 μg/ml, marked 20 and 50, respectively, did not significantly alter the amount of OS bound or internalized by ARPE-19 cells. Control cells received Fab fragments of nonimmune IgG at 50 μg/ml. (B) Fab fractions of rat CD36 IgG at 50 μg/ml, marked 50, decreased the OS internalization index of rat RPE-J cells to 54% of the index of cell receiving preimmune Fab fractions (Student's *t* test $P < 0.005$). Changes in bound OS were not significant. Cross-linking of CD36 Fab at 20 and 50 μg/ml, marked 20 and 50, using goat anti–rabbit Fab IgG at 10 μg/ml reversed the effect of CD36 Fab alone. Using Student's *t* test, these changes were significant with $P < 0.005$. n.i., nonimmune.

tially restored the stimulatory effect of CD36 Abs increasing the amount of internalized OS by 2.6-fold as compared with cells treated with CD36 Fab fragments alone or by 34% compared with control cells treated with preimmune Fab fragments plus cross-linker (Fig. 4 B). In the absence of cross-linker, anti–rat CD36 Fab fragments reduced OS internalization by 46% (Fig. 4 B). Addition of cross-linking secondary Abs to FA6–152 Fab fragments did not rescue the Ab's effect on OS uptake suggesting that the generation of Fab fragments may have altered antigen recognition by the Ab. Taken together, these results suggested that stimulation but not inhibition of the RPE's internalization rate required bivalent CD36 Abs.

CD36 Abs Act during OS Internalization, Independently of the OS Binding Receptor αvβ5 Integrin. Interestingly, CD36 Abs affected OS internalization only at time points later than 2 h after OS challenge even though Abs were added to the cells with OS challenge. Therefore, we determined the window of time during which addition of stimulating concentrations of CD36 Ab was effective. To this end, we challenged RPE-J cells with FITC-OS for 2 h in the presence or absence of Ab. After 2 h we removed unbound OS and allowed phagocytosis to proceed in the presence or absence of Abs to determine their effect on the internalization rate of OS prebound to the RPE surface. As expected, RPE cells bound similar numbers of OS regardless of Ab treatment during the first 2 h of the experiment

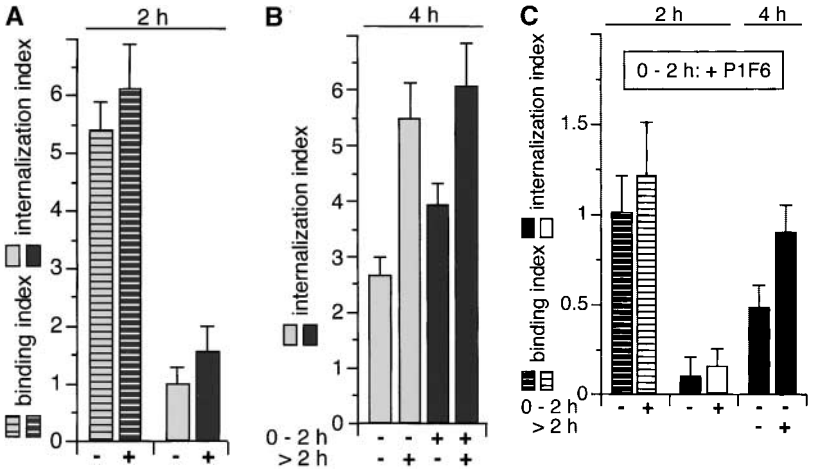


Figure 5. Addition of CD36Ab after OS binding to RPE is necessary and sufficient to increase the rate of OS internalization by RPE, independently of the OS binding receptor αvβ5. Differentiated monolayers of RPE-J cells were challenged for 2 h with FITC-OS in the presence of 50 μg/ml preimmune IgG or 50 μg/ml rat CD36 IgG. After 2 h, some samples were fixed to determine binding and internalization indices as shown in A. Striped bars, OS binding; filled bars, OS internalization; −, preimmune IgG control; +, CD36 IgG. (B) Identical samples as in A were washed with assay buffer 2 h after OS addition to remove unbound OS and further incubated in presence of either preimmune (−) or CD36 IgG (+). B shows the internalization indices of these samples determined 4 h after initial OS challenge (2 h after the removal of unbound OS). (C) We repeated the experiments shown in A and B but added αvβ5 inhibiting Ab P1F6 to all samples during the 2 h of OS challenge. P1F6 reduced OS binding at 2 h equally well in the presence of preimmune IgG, (−, dark striped bars) or of CD36 IgG (+, light striped bars). Internalization indices for both conditions were insignificant after 2 h (2 h, dark and light filled bars). Samples that were incubated with OS and P1F6 for 2 h were washed and incubated for another 2 h in the presence of preimmune (−) or CD36 IgG (+). Internalization indices that were determined after a total time of incubation of 4 h demonstrated that CD36 IgG accelerated internalization of OS prebound in the presence of P1F6 (4 h; −, preimmune IgG, +, CD36 IgG added after removal of unbound OS at 2 h). Note that the y axis scale in C is different from that in A and B. Values in A–C represent average OS indices ± SD ($n = 3$). (B and C). Statistical evaluation using Student's *t* test determined $P < 0.001$ for all changes compared with the appropriate control condition that were discussed as significant in the text. Note that all cells regardless of treatment completed internalization of prebound OS within 6 h after initial OS challenge (data not shown). Between the removal of unbound OS at 2 h and the termination of the experiment, the total amount of OS detected in each sample remained constant (data not shown). Thus, surface-bound OS did not dissociate from RPE cells and RPE cells did not degrade phagocytosed OS between 2 and 6 h after initial OS challenge.

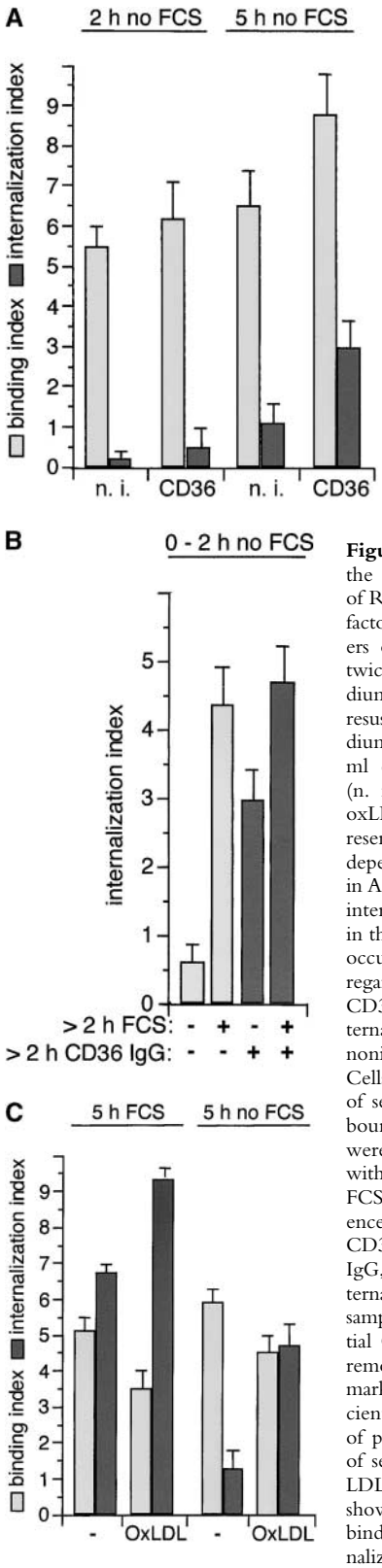


Figure 6. CD36 Abs activate the OS internalization function of RPE in the absence of soluble factors. Differentiated monolayers of RPE-J cells were rinsed twice with warm serum free medium before challenge with OS resuspended in serum free medium in the presence of 50 μg/ml either of nonimmune IgG (n. i.), CD36 IgG (CD36), or oxLDL for 2 h or 5 h. Bars represent averages ±SD of three independent experiments. (A) Bars in A show binding (light bars) and internalization (dark bars) of OS in the absence of serum. Binding occurred in the absence of serum regardless of the stimulation of CD36. CD36 Abs increased internalization compared with nonimmune IgG ($P < 0.02$). (B) Cells received OS in the absence of serum for 2 h, at which unbound OS were removed. Cells were further incubated with or without 3% FCS (labeled: >2 h FCS, + or −) and in the presence of either preimmune or CD36 IgG (labeled: >2 h CD36 IgG, − or +). B shows the internalization indices of these samples determined 5 h after initial OS challenge (3 h after the removal of unbound OS). Remarkably, CD36 Abs were sufficient to accelerate internalization of prebound OS in the absence of serum ($P < 0.001$). (C) OxLDL (but not LDL, data not shown) had no effect on OS binding but increased OS internalization in the presence or absence of serum, as indicated in the Figure, during 5 h of coincubation with OS ($P < 0.005$). Thus, this multivalent CD36 ligand mimicked the effect observed with intact Ab.

(Fig. 5 A, striped bars). Strikingly, addition of Ab after 2 h to cells previously unexposed to Ab was equally effective in increasing the internalization rate of surface-bound OS as the addition of Ab during both OS binding and internalization (Fig. 5 B, compare −/+ and +/+). Removal of Ab after the initial 2 h of OS binding resulted in the same internalization rate of bound OS as the absence of Ab throughout the experiment (Fig. 5 B, compare +/− and −/−). These data clearly demonstrated that the presence of stimulating concentrations of CD36 Ab during the internalization phase of OS phagocytosis was necessary and sufficient to accelerate internalization of bound OS by RPE.

Previously, we reported that inhibition of αvβ5 integrin using the heterodimer-specific blocking Ab P1F6 reduced the number of OS bound by RPE during 2 h of OS challenge to 16% of control cells (6). To test whether CD36 Abs affected internalization of OS bound when αvβ5 was blocked we performed coincubation experiments with both CD36 and αvβ5 integrin Abs. As expected, P1F6 drastically reduced OS binding by RPE (Fig. 5, compare striped bars of A and C). CD36 IgG did not change the amount of OS bound in the presence of αvβ5 Ab (Fig. 5 C, striped bars, 2 h) but the addition of CD36 IgG after the removal of unbound OS again accelerated internalization of the small number of OS bound by RPE during the initial 2-h period when αvβ5 was inhibited (Fig. 5 C, black bars, 4 h). These results suggested that CD36 function in OS internalization was independent of αvβ5 function in OS binding.

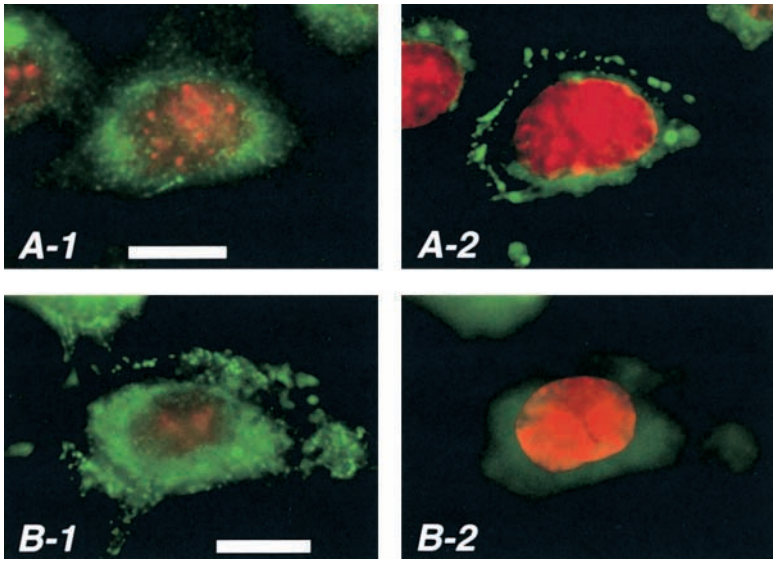
CD36 Abs or Oxidized LDL Activate OS Internalization by RPE in the Absence of Serum. Our results strongly implied a regulatory role of CD36 in internalization of bound OS likely involving CD36 dimerization. Therefore, we hypothesized that activation of CD36 using stimulating concentrations of intact CD36 Ab may be sufficient to trigger internalization of bound OS. To test this hypothesis, we challenged confluent RPE-J cells with OS under conditions that selectively inhibit internalization by RPE in culture, i.e., low temperature and the absence of serum.

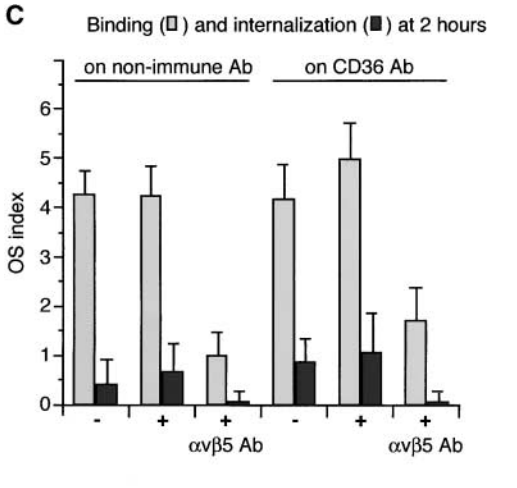
Addition of CD36 Ab had no effect on OS binding or on OS internalization by RPE-J cells kept at 18°C (data not shown).

In the absence of serum, OS remained externally bound to the surface of control RPE-J cells during 5 h of OS challenge. Addition of CD36 Ab at the time of OS challenge increased OS internalization 2.8-fold compared with cells receiving preimmune IgG (Fig. 6 A). To study whether CD36 Ab could activate internalization of OS prebound by RPE in the absence of serum and Ab, we challenged RPE-J cells for 2 h with OS in the absence of serum, removed unbound OS, and continued the incubation in the presence or absence of CD36 Abs and of heat-inactivated serum. RPE-J cells promptly internalized bound OS when serum was replenished but not when serum was omitted (Fig. 6 B, +/− and −/−). Strikingly, cells that received CD36 Abs after OS binding largely internalized prebound OS even in the absence of serum (Fig. 6 B, −/+). Furthermore, addition of oxidized LDL (oxLDL), a multivalent

ligand for CD36 (21) had the same effect as addition of CD36 Ab, increasing OS internalization in the presence of serum and activating OS internalization in the absence of serum (Fig. 6 C). Lipoprotein-deficient serum fully retained the capacity of complete serum to initiate OS internalization (data not shown). Thus, low concentrations of oxLDL present in serum were not responsible for the serum effect on OS phagocytosis. However, cross-linking of CD36 using Abs or oxLDL partially substituted for unknown serum factor/s whose presence is necessary for the initiation of OS internalization by RPE in culture.

CD36 Ligation at the Basal Surface Induces Internalization of OS Bound to αvβ5 at the Apical Surface of RPE. Our results suggesting that CD36 ligation regulated the mechanism used by RPE to internalize OS fit two different scenarios: (i) ligated CD36 may interact directly with bound OS, with αvβ5 (possibly activating αvβ5's internalization function), or with other components of the internalization machinery at the apical surface of RPE, and (ii) ligated CD36 may initiate a cytoplasmic signaling mechanism that in turn activates components of the internalization machinery of RPE. To determine whether CD36 function required direct interaction of CD36 with the apical phagocytic machinery of RPE we tested the effects of CD36 ligation at the RPE basal surface on OS phagocytosis. Initial experiments determined that RPE-J cells adhered and spread equally well and appeared of similar morphology on CD36 and on nonimmune Ab-coated coverslips (data not shown). Regardless of their substrate, RPE-J cells required a minimum of 6 h to attach and spread. At this time, at which we used the cells for experiments, immunofluorescence labeling showed that the tight junction component ZO-1 was distributed diffusely in the cytoplasm differently from the circumferential localization characteristic for ZO-1 in polarized epithelia (data not shown). This demonstrated that cells did not possess functional tight junctions and, thus, lacked a plasma membrane protein permeability barrier. On nonimmune Ab, CD36 localized diffusely to the basal surface as well as to plasma membrane facing the medium (Fig. 7, A1 and A2). On CD36 Ab, CD36 localized predominantly to the basal surface in a punctate staining pattern confirming that immobilized CD36 Ab bound and re-





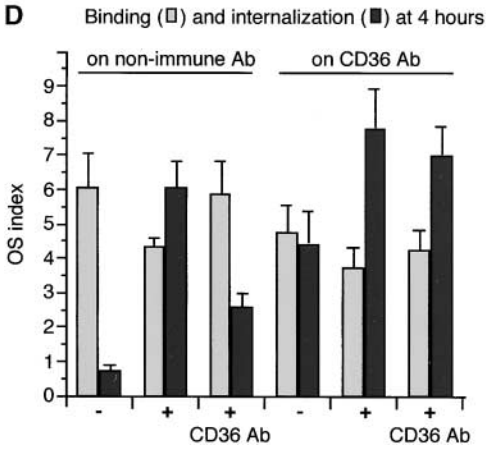


Figure 7. CD36 Ab binding of CD36 at the RPE basal surface stimulates internalization of OS bound to αvβ5 at the apical surface. RPE-J cells adherent on coverslips coated with nonimmune Ab (A) or CD36 Ab (B) for 6 h were stained with murine CD36 Ab. 2 x-y sections of each field are shown, corresponding to the basal surface plane (A-1, B-1) and to a plane 6 μm above the basal surface (A-2, B-2), representing a subapical-lateral plane of an RPE-J cell. Cover glass staining caused by coating Ab was subtracted from basal fields as background. Total height of cells seeded on coated overslips was ~8–10 μm (data not shown). While most RPE cells were in contact with neighboring cells 6 h after seeding, cells exhibited membrane protrusions (A2, B1) and had not established a polarized phenotype. Green signals shows CD36 labeling while red nuclei stain serves as reference. Scale bars: 10 μm. In RPE-J cells on control Ab, CD36 localized to both basal and subapical plasma membrane (A-1, A-2). In contrast, in cells on CD36 Ab, CD36 was prominent at basal attachment sites (B-1) and mostly absent from the subapical plane (B-2) suggesting that the CD36 Ab trapped CD36 at the glass surface. (C) During 2 h of OS challenge, RPE-J cells on control or on CD36 Ab bound FITC-OS normally, independent of the presence (+) or absence (−) of FCS. αvβ5 inhibitory Ab P1F6 at 50 μg/ml interfered with OS binding (+, αvβ5 Ab) Shown are average OS indices ± SD ($n = 3$). (D) During 4 h of OS challenge, cells on nonimmune Ab internalized OS in the presence (+) but not the absence (−) of serum. In contrast, cells on CD36 Ab internalized increased numbers of OS in the absence of serum (−) (averages ± SD, $n = 4$, $P < 0.005$). Soluble CD36 Ab at 100 μg/ml that reduced internalization by control cells (averages ± SD, $n = 3$, $P < 0.01$) had no effect on internalization by cells whose CD36 was trapped at the basal surface.

distributed CD36 (Fig. 7, B1 and B2). RPE-J cells on different Abs, via αvβ5, bound equal numbers of OS within 2 h (Fig. 7 C). However, attachment of RPE to CD36 Ab but not to nonimmune Ab promoted OS internalization in the absence of serum (Fig. 7 D, −). Cells seeded on either immobilized Ab internalized OS in the presence of serum (Fig. 7 D, +) but, importantly, inhibitory concentrations of soluble CD36 Ab added during OS challenge reduced OS internalization by cells on nonimmune Ab but had no effect on OS internalization by cells attached to CD36 Ab (Fig. 7 D, +, CD36 Ab). These data demonstrated that the effect of CD36 ligation was independent of interaction with OS or with the phagocytic machinery of RPE at the apical surface including αvβ5 integrin. This strongly supported hypothesis (2) that CD36 acted primarily as a signaling molecule in RPE phagocytosis whose ligation activated the RPE internalization machinery indirectly.

Discussion

Several studies have previously documented that CD36 participates in clearance phagocytosis by cell types as different as human monocytes and dendritic cells (15, 16, 35), rat RPE (9), and *Drosophila* hemocytes (36). Despite its obvious importance, the specific role of CD36 in clearance phagocytosis has still remained unclear, for any phagocyte. In this study, we elucidate the contribution of CD36 to OS phagocytosis by the RPE.

(i) CD36 Function in OS Internalization by RPE Is Independent of αvβ5 Integrin Function. Maximal inhibition of αvβ5 integrin using blocking Abs decreases OS binding to 16% of the OS binding of untreated cells. It is possible but it has not yet been investigated that the remaining OS bind to a different, unidentified OS binding receptor that may play a minor role in normal RPE binding of OS in parallel with αvβ5. CD36 Abs had no effect on OS binding but increased the internalization rate both for OS bound to αvβ5 and for OS bound to RPE when αvβ5 was blocked. Thus, CD36 Abs accelerate OS internalization independent of the OS binding receptor available to RPE, and of the total number of OS bound by RPE.

(ii) Different Concentrations of CD36 Abs Accelerate or Inhibit OS Internalization at the Time when Internalization Occurs in Control Cells. Bivalency Is Necessary for the Increasing but not the Decreasing Effect of CD36 Abs on the Rate of OS Internalization. Stimulating concentrations of bivalent Ab likely form additional CD36 receptor dimers compared with normal assay conditions, while inhibitory Ab concentrations provide excess antigen-binding sites preventing CD36 dimerization normally induced by CD36 receptor-ligand binding. This agrees well with earlier reports showing that CD36 dimer assembly correlates with its signaling function (37, 38). Furthermore, Ab cross-linking of CD36 substitutes for the antiangiogenic properties of the CD36 ligand thrombospondin-1 (33).

The slow onset of internalization by RPE in culture without the need for protein synthesis (18) suggests that cultured RPE or experimental OS lack yet unknown properties that accelerate ingestion. CD36 ligation does not shorten the lag phase before onset of internalization characteristic for phagocytosis by RPE in culture but increases the internalization rate at exactly the time when internalization occurs in control cells. Thus, OS uptake by RPE in culture is not slower than uptake by RPE in the eye due to lack of CD36 ligation.

(iii) CD36 Ligation by Ab or oxLDL Substitutes for Soluble Factors Required for Phagocytosis by RPE in Culture and Present In Serum. Expression and Availability of CD36 on the Phagocyte and Production or Exposure of CD36 Ligand, Soluble or Insoluble, May Determine the Individual or Collective Rate of Internalization of any Particle and Phagocyte Combination. When we selectively inhibited internalization by omitting serum, modulation of CD36 had no effect on OS binding, but increased internalization. Thus, CD36 stimulation using cross-linking Abs or the multivalent ligand oxLDL is sufficient to activate the internalization machinery of RPE. Our results suggest that nonlipoprotein CD36 ligands in serum cross-link CD36 receptors at the RPE surface and thereby induce OS internalization by RPE in culture. Since CD36 localizes to the apical surface of polarized RPE cells in culture and in the retina (9) serum is only effective if added to the apical surface, as demonstrated earlier (20). One candidate ligand for CD36 abundant in serum is thrombospondin-1. Furthermore, thrombospondin-1, which is synthesized by human RPE in situ and in culture (39), may cluster CD36 in the subretinal space to regulate the internalization mechanism of RPE in vivo.

Importantly, these results imply that in any interaction of phagocytes with phagocytic particles, the number of CD36 receptors exposed at the phagocytic cell surface in relation to the density of CD36 ligand determines a specific rate of particle internalization. If concentrations and availability of soluble CD36 ligands are controlled by the phagocytes, the internalization rate may be largely independent of the individual nature of each particle to be phagocytosed. One such soluble ligand may be thrombospondin-1 that is secreted during phagocytosis of apoptotic neutrophils by monocyte derived macrophage phagocytes (15). In contrast, if CD36 ligands are bound to or part of the surface of the phagocytic particle, the concentration or exposure of ligand by each individual particle likely contributes to the regulation of its individual engulfment rate. One such surface-bound ligand may be phosphatidylserine exposed by cells undergoing apoptosis (14).

(iv) CD36 Ligation Is Equally Effective at the Nonphagocytic Surface of RPE Suggesting a Signaling Role of CD36. Recruitment of CD36 to the basal attachment site of RPE cells by immobilized CD36 Ab activated the internalization mechanism of RPE substituting for CD36 ligation by soluble serum factors. Using a similar strategy, Maxeiner et al. demonstrated that CD36 ligation by immobilized ligand oxLDL stimulates H_2O_2 secretion by monocytes probably by activating CD36 signaling (40). We conclude that

CD36 dimers do not regulate internalization by forming or directly interacting with the RPE internalization machinery. We propose that, instead, CD36 dimerization activates a cellular signaling pathway whose target is the internalization mechanism of RPE.

Our findings provide strong evidence that dimerization of CD36 receptors at the surface of RPE phagocytes regulates the rate of OS internalization by the RPE. Using this knowledge, we will continue our studies to identify the endogenous ligand that activates CD36 in RPE in the retina. The results presented here will also form a basis for future studies on other phagocytes that employ CD36 to test whether CD36 plays an equivalent role in phagocytic mechanisms other than the RPE's.

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