

The $\alpha 5\beta 1$ Integrin Mediates Elimination of Amyloid- β Peptide and Protects Against Apoptosis

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Abstract. The amyloid- β peptide (A β) can mediate cell attachment by binding to $\beta 1$ integrins through an arg-his-asp sequence. We show here that the $\alpha 5\beta 1$ integrin, a fibronectin receptor, is an efficient binder of A β , and mediates cell attachment to nonfibrillar A β . Cells engineered to express $\alpha 5\beta 1$ internalized and degraded more added A $\beta 1$ -40 than did $\alpha 5\beta 1$ -negative control

cells. Deposition of an insoluble A $\beta 1$ -40 matrix around the $\alpha 5\beta 1$ -expressing cells was reduced, and the cells showed less apoptosis than the control cells. Thus, the $\alpha 5\beta 1$ integrin may protect against A β deposition and toxicity, which is a course of Alzheimer's disease lesions.

INTEGRIN-mediated cell adhesion is necessary for the survival of many types of cells, and loss of adhesion causes apoptosis (reviewed in Frisch and Ruoslahti, 1997). The $\alpha 5\beta 1$ integrin may have a particularly prominent antiapoptotic effect because $\alpha 5\beta 1$ is the only integrin that protects cells from apoptosis in serum-free cultures (Zhang et al., 1995; O'Brien et al., 1996). $\alpha 5\beta 1$ -mediated adhesion upregulates the antiapoptosis protein Bcl-2 (Zhang et al., 1995), and $\alpha 5\beta 1$ is one of a few integrins that activates the signaling protein Shc (Wary et al., 1996). These signaling events may partly explain its antiapoptotic effects.

$\beta 1$ integrins have been shown to mediate cell adhesion to the amyloid beta (A β)¹ protein, and $\alpha 5\beta 1$ has been proposed to be the integrin responsible for the A β binding (Ghiso et al., 1992). The amino acid sequence arg-his-asp (RHD) has been pinpointed as the integrin recognition site in A β (Ghiso et al., 1992; Sabo et al., 1995). This sequence resembles the general integrin recognition sequence RGD present in many extracellular matrix proteins (Ruoslahti, 1996a).

A β is a 39–42 amino acid protein derived from proteolytic cleavage of a larger membrane-spanning glycopro-

tein, the amyloid precursor protein (APP; Kang et al., 1987). A β forms fibrillar aggregates that can cause cell death by apoptosis (Loo et al., 1993; Pike et al., 1993; Lorenzo and Yanker, 1994). Enhanced deposition of A β matrix within the cortex, hippocampus, and vasculature of the brain correlates with neuronal cell death and ultimately dementia in Alzheimer's disease (AD; reviewed by Selkoe, 1994). Two predominant forms of A β (1–40 and 1–42) exist in AD that differ by two amino acid residues at the hydrophobic COOH terminus, a domain that is required for nucleation-dependent fibril formation (Jarret et al., 1993). The A $\beta 1$ -40 form has a slower rate of fibril formation in vitro than the A $\beta 1$ -42 form (Jarret et al., 1993).

There is evidence for three mechanisms of A β accumulation: overproduction of A β , production of longer forms of A β (which aggregate more), and impaired clearance of A β . The clearance pathways for fibrillar and soluble A β are incompletely known. Two cell surface receptors are known to bind A β . The scavenger receptor present on glial cells binds specifically to fibrillar A β , and appears to mediate clearance of small fibrillar A β aggregates in vitro (Paresce et al., 1996; Khoury et al., 1996). The receptor for advanced glycation end products binds both the soluble and fibrillar forms of A β , and may mediate some of the cytotoxic effects of fibrillar A β (Yan et al., 1996).

Because $\alpha 5\beta 1$ may also be an A β receptor, and because $\alpha 5\beta 1$ and A β have apparently contrasting effects on apoptosis, we sought to determine whether $\alpha 5\beta 1$ is indeed an A β -binding integrin and, if so, what effect it might have on the metabolism of A β and on cell survival. We show here that nonfibrillar A β binds to the $\alpha 5\beta 1$ integrin, and that

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1. *Abbreviations used in this paper:* A β , amyloid β peptide; AD, Alzheimer's disease, LDH, lactate dehydrogenase; RHD, arg-his-asp.

this interaction promotes clearance of A β by cultured cells, reducing the formation of an insoluble A β fibrillar matrix and counteracting the toxic effects of the A β matrix. These results suggest a new function for $\alpha 5\beta 1$ as a binder of A β and a regulator of brain cell survival.

Materials and Methods

Cells

The human neuroblastoma cell line (IMR-32) was obtained from the American Type Culture Collection (Rockville, MD). The CHO-B2 cells deficient in $\alpha 5\beta 1$ were from Dr. Rudolf Juliano (School of Medicine, University of North Carolina, Chapel Hill, NC; Schreiner et al., 1989). All cells were maintained in α -MEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS and glutamine/pen-strep (Irvine Scientific, Santa Ana, CA). G418 (GIBCO BRL, Gaithersburg, MD) was added to the media of transfected cells at a concentration of 250 μ g/ml.

Reagents

Amyloid beta 1-40 peptide (A β) was synthesized as previously described (Nordstedt et al., 1994). A β was also purchased from a commercial source (Synthetic Amyloid Beta peptide 1-40; Bachem, Torrance, CA). A β 1-40 from both sources was examined for cell adhesion activity. Two out of the three Bachem lots tested showed adhesive activity (lots zn571 and wm365), while lot zn327 was not active. For water-free storage to prevent aggregation of A β into its fibrillar form, the peptide was dissolved and stored in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Fluka Chemika, Neu-Ulm, Switzerland). Before use, the peptide was lyophilized from HFIP, dissolved in sterile distilled water at 1 mg/ml, and tested immediately. The control peptide, A β 40-1, was purchased from Bachem, solubilized in water at 1 mg/ml, and tested immediately. Fibronectin was purchased from Chemicon International, Inc. (Temecula, CA), and vitronectin was purified as described (Yatohgo et al., 1988). Purified anti-human $\alpha 5$ integrin monoclonal antibody (P1D6; Calbiochem-Novabiochem Corp., La Jolla, CA; Wayner et al., 1988) and purified mouse IgG (Sigma Chemical Co.) were used at a concentration of 50 μ g/ml.

Transfection

The CHO-B2/ $\alpha 5\beta 1^+$, CHO-B2/ $\alpha v\beta 1^+$, and IMR-32/ $\alpha 5\beta 1^+$ cells were generated by introducing cDNAs coding for the $\alpha 5$ and αv integrin subunits into $\alpha 5\beta 1$ -deficient CHO-B2 and IMR-32 cells (Schreiner et al., 1989; Bauer et al., 1992; Zhang et al., 1993, 1995). Transfectants expressing the integrin were cloned and expanded (Zhang et al., 1993; Zhang et al., 1995). CHO-B2 and IMR-32 control cells received the empty vector.

Integrin Analysis

Integrin expression of IMR-32 and CHO transfectants was analyzed by FACS using monoclonal antibodies against human $\alpha 5$ (P1D6), αv (L230), and $\beta 1$ (P4C10). FITC-conjugated goat anti-mouse antibody (Sigma Chemical Co.) was used as the secondary antibody. The same integrin antibodies were used to block integrin function in other experiments.

Cell Adhesion to Nonfibrillar A β 1-40

The cell attachment assay and the use of antibodies and peptides as inhibitors of adhesion have been described previously (Zhang et al., 1993; Matter and Laurie, 1994). Microtiter plates coated overnight at room temperature with nonfibrillar A β 1-40 peptide, control A β 40-1 peptide, or fibronectin were blocked with 1% BSA for 30 min at room temperature, the wells were rinsed once with PBS (pH 7.4), and cells were subsequently added (2×10^5 cells/well) in serum-free media and incubated for 60 min (37°C). Inhibition studies were performed by preincubating cells with antibody for 30 min (37°C; gentle agitation every 10 min), and then cells including antibodies were added to the coated wells. After a 60-min incubation at 37°C, plates were gently washed four times with PBS, fixed with 1% glutaraldehyde (Sigma Chemical Co.), PBS-washed once, stained with 0.5% crystal violet, 20% MEOH, washed under running distilled water, solubilized in 0.1 N sodium citrate, 50% ETOH, and read on an ELISA plate reader (Molecular Devices Corp., Sunnyvale, CA) using the 590-nm filter.

Adhesion assays with fibrillar A β 1-40 were performed as above. Before the adhesion assay, soluble A β 1-40 was incubated at 4°C for 96 h to allow self-aggregation of A β 1-40 into its fibrillar form (Jarret et al., 1993). Coating efficiency was measured by coating microtiter wells with either soluble [125 I]A β 1-40 or preaggregated [125 I]A β 1-40 at room temperature overnight. Nonbound peptide solution was removed, and the well and the nonbound peptide solution were counted. Both forms of A β 1-40 bound to the wells with an efficiency of $\sim 70\%$.

Immunostaining of A β Fibrillar Matrix

Cells were plated on four-well Permanox™ plastic slides (Nunc Inc., Naperville, IL) at 50,000 cells/well. 6 h after plating, the media was replaced with media containing A β 1-40 peptide (100 μ g/ml) and incubated for 72 h at 37°C. The cultures were washed with PBS and fixed in PBS containing 3.7% paraformaldehyde and 10 mM sucrose, pH 7.4, for 30 min at room temperature. The cultures were then blocked with 1% BSA/PBS and stained with a polyclonal rabbit anti-human A β 1-40 peptide antibody (Chemicon International, Inc.) for 2 h, followed by goat anti-rabbit FITC-labeled IgG (Sigma Chemical Co.) secondary antibody. After antibody treatment, coverslips were mounted with Vectashield mounting medium (Vector Labs., Inc., Burlingame, CA) and analyzed under a fluorescent confocal microscope.

Analysis of A β in Matrix Deposition with Radiolabeled [125 I]A β

125 I-labeled A β 1-40 peptide was purchased as a lyophilized powder (25 μ Ci) from Nycomed Amersham, Inc. (Princeton, NJ). The powder was solubilized in sterile water and immediately added to 24-well culture dishes at a concentration of 2 ng/well. The specific activity of the 125 I-labeled A β 1-40 peptide was 2×10^6 μ Ci/mmol.

Insolubilization of A β was analyzed using 125 I-labeled A β 1-40 peptide as described previously for fibronectin matrix assembly (McKeown-Longo and Mosher, 1985; Morla and Ruoslahti, 1992). Cells were plated at 10^5 cells/ml (IMR variants) or 0.5×10^5 cells/ml (CHO variants) into 24-well tissue culture plates in media containing 10% serum. Media was replaced 6 h after plating with media containing [125 I]A β 1-40 and 10% serum. Cells were cultured for 72 h at 37°C. The media was then removed, the wells were washed three times with PBS, and 5 \times SDS sample buffer (0.5M Tris pH 6.8, glycerol, 10% SDS, 0.5% bromophenol blue) was used to solubilize the [125 I]A β matrix in each well.

For antibody inhibition experiments, cells were plated as above. 6 h after plating, the media was replaced with media containing the appropriate antibody and 10% serum. 125 I-labeled A β 1-40 peptide (2 ng/well) was added to the antibody-containing media and incubated for 72 h at 37°C. The cells were then processed as above.

Internalization and Degradation of [125 I]Soluble A β 1-40

Internalization of A β 1-40 added to cell layers was measured as described (Duckworth et al., 1972; McDermott and Gibson, 1997). Subconfluent cells were trypsinized and plated onto 24-well plates. Media was replaced 6 h after plating with [125 I]A β 1-40 (2 ng/ml). The cells were incubated for 1 h with [125 I]A β 1-40, the media was removed, cells were washed five times with PBS, and serum-containing media containing no A β 1-40 was added. The cells were cultured for 1 to 12 h at 37°C, washed three times with PBS, detached by EDTA, washed twice with PBS, lysed in 100 μ l of 1% NP40 buffer for 10 min at 4°C, and lysate-analyzed for radioactivity.

For TCA precipitations, the cells were cultured for 72 h with [125 I]A β 1-40 at 37°C, washed three times with PBS, detached by EDTA, washed twice with PBS, and lysed in 100 μ l of 1% NP40 buffer for 10 min at 4°C. BSA/PBS (100 μ l, 1%) was added to the samples, the samples were vortexed, and 1.6 ml of TCA (12.5% wt/vol) was added with vortexing. The samples were centrifuged at 2,000 rpm for 10 min at 4°C, and the supernatant and pellet were collected for radioactive counting.

Secretion of 125 I-Labeled A β 1-40

Subconfluent cells were detached with trypsin, washed once with media, and plated at 10^5 cells/ml in 24-well plates. 6 h after plating, media was replaced with 2 ng/ml of [125 I]A β 1-40 in serum-containing media and incubated for 1 h at 37°C. The radiolabeled media was removed, and cells were

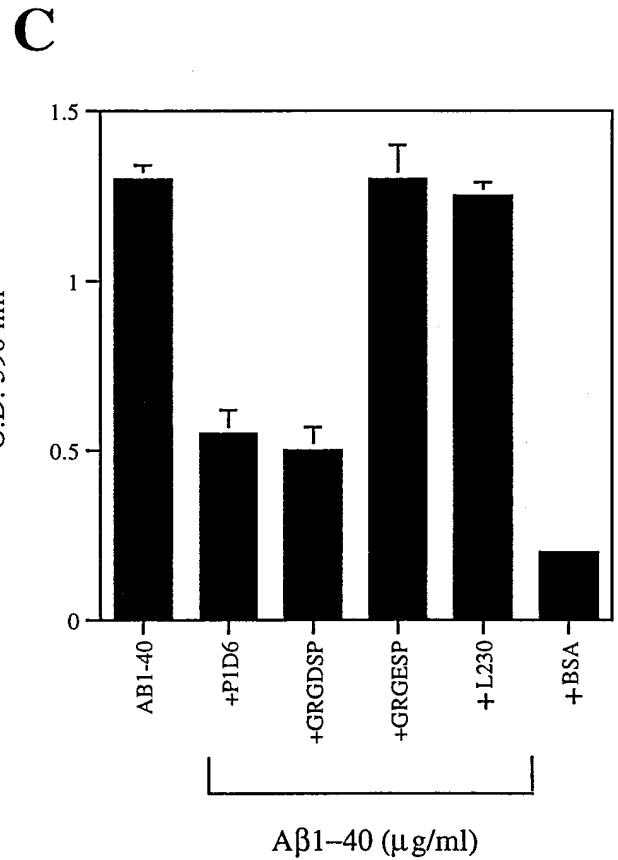
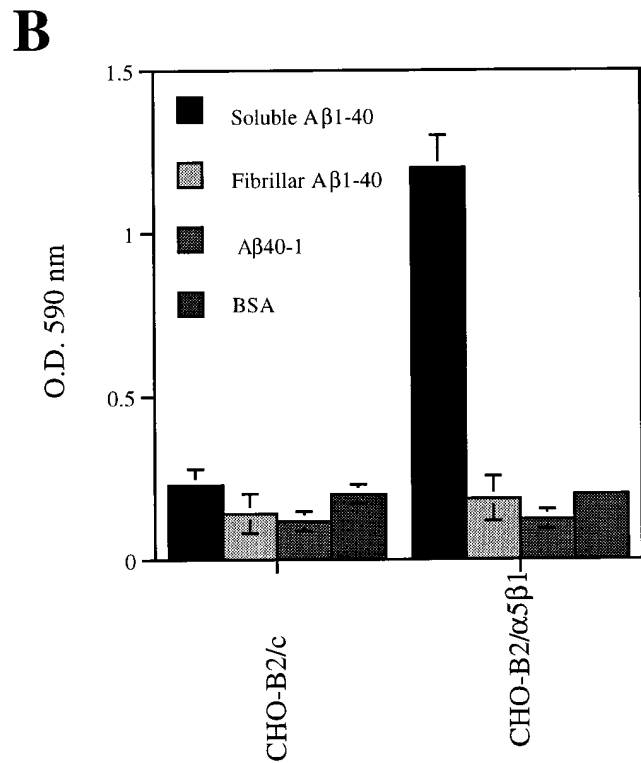
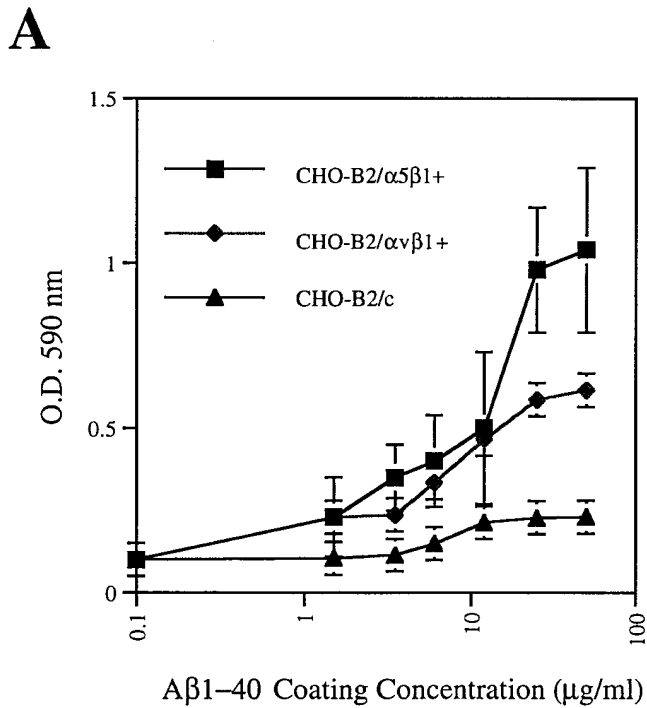


Figure 1. Integrins mediate CHO cell adhesion to nonfibrillar A β . Adhesion of CHO cells to coated A β 1-40 was measured. The cells were transfected with human α 5 or α v integrin subunit cDNA. (A) The cells were seeded onto various concentrations of coated A β 1-40. (B) Cells were plated onto A β 1-40 coated in its soluble form, A β 1-40 coated after self-aggregation into its fibrillar form, or on the control peptide A β 40-1. (C) Cells were preincubated with either the blocking monoclonal antibodies to human α 5 (PID6), human α v (L230), the integrin-binding peptide GRGDSP, or the control peptide GRGESP, and then seeded onto coated soluble A β 1-40. After a 60-min incubation at 37°C, attached cells were quantitated. Values represent the mean \pm SD; $n = 9$.

washed five times in PBS before serum-containing media containing no A β was added to each well. At designated time points, 100 μ l of media was collected, and [125 I] was measured.

Apoptosis and Cell Viability Assays

The apoptotic effect of fibrillar A β was determined using the Apoptag Plus In Situ Apoptosis KitTM (Oncor, Inc., Gaithersburg, MD) that detects the 3'-OH region of cleaved DNA. Cells were plated on eight-chamber tissue culture glass slides (Miles Scientific Laboratories, Inc., Naperville, IL), and 6 h after plating the media was replaced with media containing either A β 1-40 peptide (50 μ g/ml) or A β 40-1 control peptide (50 μ g/ml) and 10% serum. Cells were cultured for 72 h at 37°C, and were then fixed in a solution containing 3.7% paraformaldehyde, 10 mM sucrose in PBS for 30 min at room temperature. Cells were stained following kit protocol, counterstained with propidium iodide/antifade solution (Oncor, Inc.), mounted, and viewed under a confocal microscope.

To measure apoptosis by nuclear fragmentation, cells were plated in wells coated with either 50 μ g/ml of fibronectin, vitronectin, or A β 1-40 for 72 h in serum-free medium. Attached and floating cells were then collected by centrifugation, washed once with PBS, fixed with 3.7% paraformaldehyde for 10 min at room temperature, and stained with 0.1 μ g of 4', 6-diamidino-2-phenylindole (DAPI) per ml in PBS. The stained cells

were washed three times with PBS and mounted onto slides for analysis under a fluorescence microscope (Zhang et al., 1995).

Cell viability was assessed in several assays. The ability of cells to take up acridine orange/ethidium bromide was measured as described (Cotter and Martin, 1996). In brief, the assay was performed in 96-well tissue culture plates containing 100 μ l media/well. Cells were plated in media containing 10% serum. 6 h after plating, the media was replaced with media containing various concentrations of the test reagents and 10% serum. The plates were incubated for 72 h at 37°C. At the 72-h time point, cells were trypsinized and resuspended in PBS at 0.5×10^6 cells/ml. 1 μ l from a solution of acridine orange (100 μ g/ml) and ethidium bromide (100 μ g/ml) was added to a 25- μ l cell suspension, incubated for 2 min at room temperature, and examined under 40 \times magnification using a Zeiss Fluorescence microscope.

Cells cultured in microtiter wells were pulsed with 25 μ l of a 2.5 mg/ml MTT stock in PBS and incubated for 4 h. Then 100 μ l of a solution containing 10% SDS, 0.01 N HCl was added, and the plates were incubated overnight (Tada et al., 1986). Absorption was read on a Vmax Microplate ReaderTM (Molecular Devices Corp., Sunnyvale, CA) using a reference wavelength of 650 nm and a test wavelength of 590 nm. Test reagents were added to media alone in order to provide a blank.

To measure lactate dehydrogenase (LDH) release from cells, the colorimetric Cytotox 96-LDH-Release AssayTM (Promega Corp., Madison, WI) was performed according to the instructions of the manufacturer.

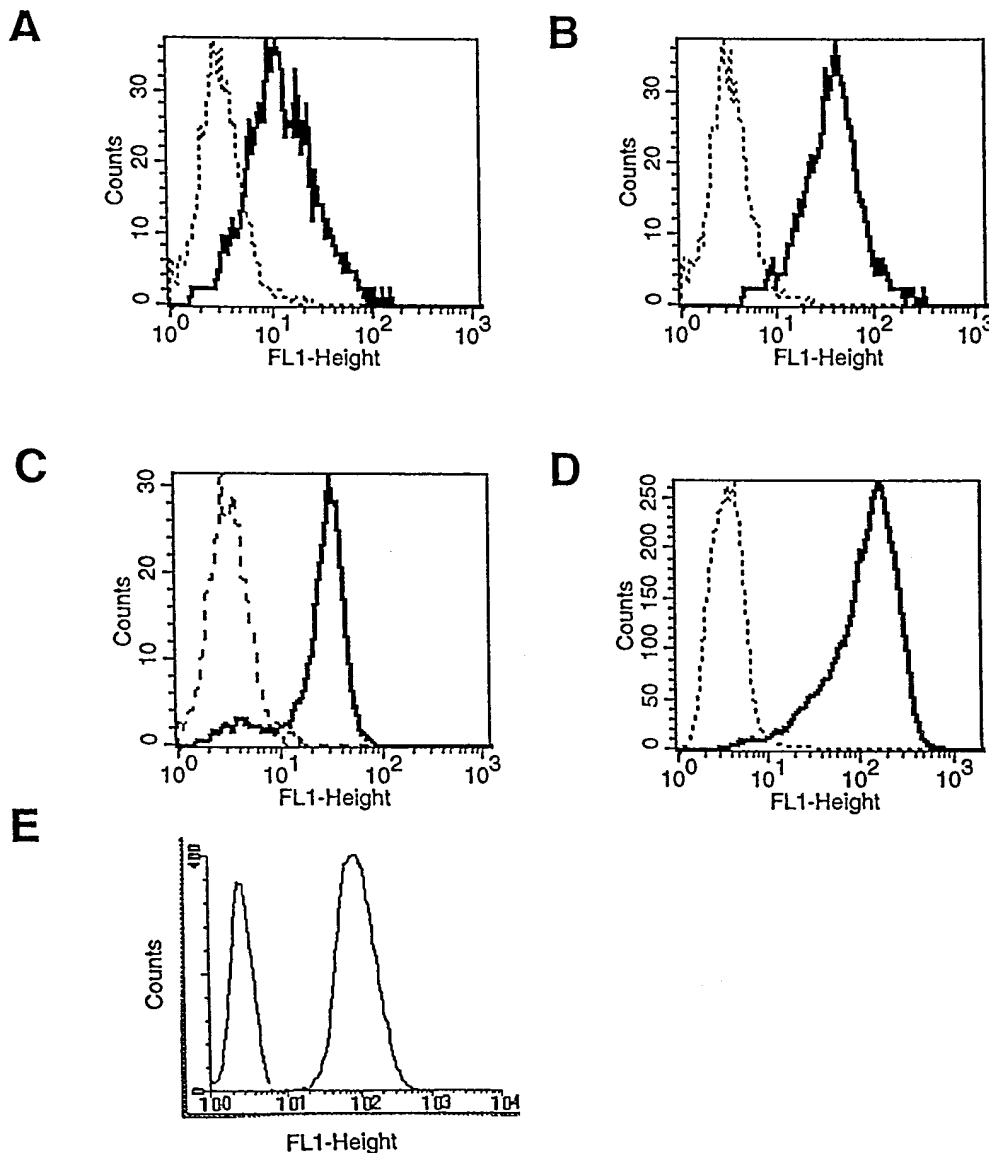


Figure 2. FACS analysis of α 5 β 1 integrin expression on IMR-32 neuroblastoma cells and CHO cells. α 5 β 1 surface expression by three IMR-32 cell clones transfected with human α 5 cDNA. IMR-32/ α 5 β 1 clone 4 (A), IMR-32/ α 5 β 1 clone 15 (B), and IMR-32/ α 5 β 1 clone 16 (C; solid lines) is compared with a vector-transfected control line IMR32/c (A and B; dashed lines), and the parental cell line IMR-32/p (C; dashed line). (D) CHO cells transfected with the human α 5 cDNA express α 5 β 1 on their surface (solid line), whereas the vector-transfected control cells (CHO-B2/c) do not (dashed line). Cells were stained with a monoclonal antibody to the human α 5 integrin subunit, followed by an FITC-labeled secondary antibody, and analyzed by FACS. (E) CHO cells transfected with the human α v cDNA express α v β 1 on their surface (solid line), whereas the vector-transfected control cells (CHO-B2/c) do not. The staining was with a monoclonal antibody to the human α v integrin subunit.

Results

The Integrin $\alpha 5 \beta 1$ Mediates Cell Adhesion to Nonfibrillar A β 1-40

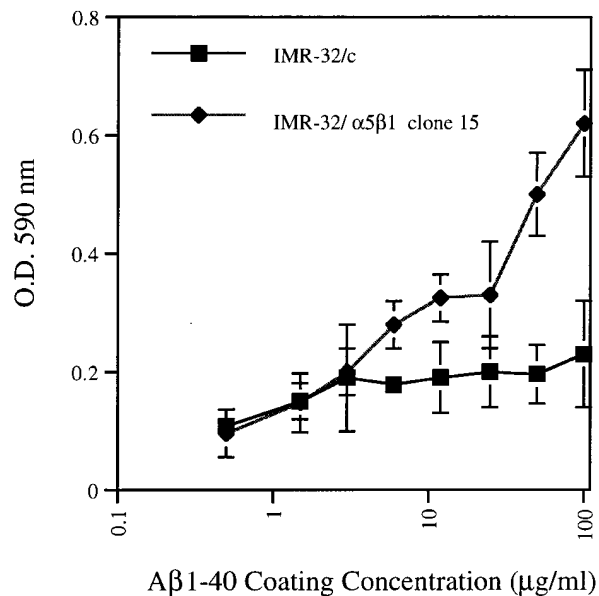
The RHD sequence in A β resembles the integrin recognition sequence RGD, and has been implicated in cell adhesion to A β via one or more of the $\beta 1$ integrins (Ghiso et al., 1992; Sabo et al., 1995). We set out to determine which of the RGD-binding integrins bind to A β . A CHO cell line deficient in $\alpha 5$ integrin subunit expression (CHO-B2) was transfected with cDNA encoding human $\alpha 5$, αv , or vector alone, and was examined for its ability to adhere to a surface coated with A β 1-40. Each of the integrin transfectants adhered to A β in a dose-dependent manner, but cells that received the vector alone attached to A β within the BSA background range (Fig. 1 A). CHO-B2/ $\alpha 5 \beta 1^+$ cells adhered strongly to A β , and CHO-B2/ $\alpha v \beta 1^+$ cells were moderately adhesive, whereas the control cells CHO-B2/c did not adhere above BSA background levels. FACS analysis indicated that CHO-B2/ $\alpha 5 \beta 1^+$ and CHO-B2/ $\alpha v \beta 1^+$ cell transfectants were similar in their expression of the transfected integrin (Fig. 2, D and E). A control peptide in which the A β sequence is inverted (A β 40-1) did not have adhesive activity with any of the cell types tested (Fig. 1 B). In addition, integrin transfectants adhered only to soluble nonfibrillar A β 1-40, and not to fibrillar A β 1-40 (Fig. 1 B). Plates were coated with equal amounts of soluble and fibrillar A β 1-40 as measured by [125 I]A β 1-40. The $\alpha 5 \beta 1$ -mediated cell adhesion to soluble A β 1-40 was inhibitable by the integrin-binding peptide GRGDSP, and by a function-blocking anti- $\alpha 5$ integrin monoclonal antibody (P1D6; Fig. 1 C), but not by the control peptide GRGESP or a monoclonal antibody to αv (Fig. 1 C). The $\alpha v \beta 3$ integrin, which also binds to RGD, does not mediate adhesion to A β because $\alpha v \beta 3$ -expressing IMR-90 cells did not adhere to A β when the $\alpha 5 \beta 1$ and $\alpha v \beta 1$ integrins were blocked with anti- $\alpha 5$ and anti- $\beta 1$ monoclonal antibodies (data not shown).

We also tested the $\alpha 5$ -negative human neuroblastoma cell line IMR-32 (Neill et al., 1994) for A β attachment with (IMR-32/ $\alpha 5 \beta 1^+$) and without (IMR-32/c) $\alpha 5$ transfection (Fig. 2 A). Three separate clones were obtained that expressed human $\alpha 5 \beta 1$ on their surface as detected by FACS analysis (Fig. 2, A–C). Each $\alpha 5 \beta 1$ -expressing clone adhered to coated A β 1-40 in a dose-dependent manner (Fig. 3 A), and cell adhesion was inhibitable by an anti- $\alpha 5$ antibody (data not shown). The control-transfected IMR-32 cells (Fig. 2, A–C) attached poorly to this substrate (Fig. 3 A). Both the transfected and control cells attached well to vitronectin (data not shown), whereas the control peptide A β 40-1 and fibrillar A β 1-40 did not promote adhesion above BSA background levels for any of the IMR-32 cell lines (Fig. 3 B).

$\alpha 5 \beta 1$ Reduces the Formation of an Insoluble A β Fibrillar Extracellular Matrix

An increase of insoluble A β fibrillar matrix is one hallmark of AD (Glennner and Wong, 1984; Masters et al., 1985). As shown above, the $\alpha 5 \beta 1$ integrin bound to coated A β with the highest avidity among the integrins we tested. Therefore, we asked whether $\alpha 5 \beta 1$ would affect the formation of an A β fibrillar matrix. Exogenous A β 1-40

A



B

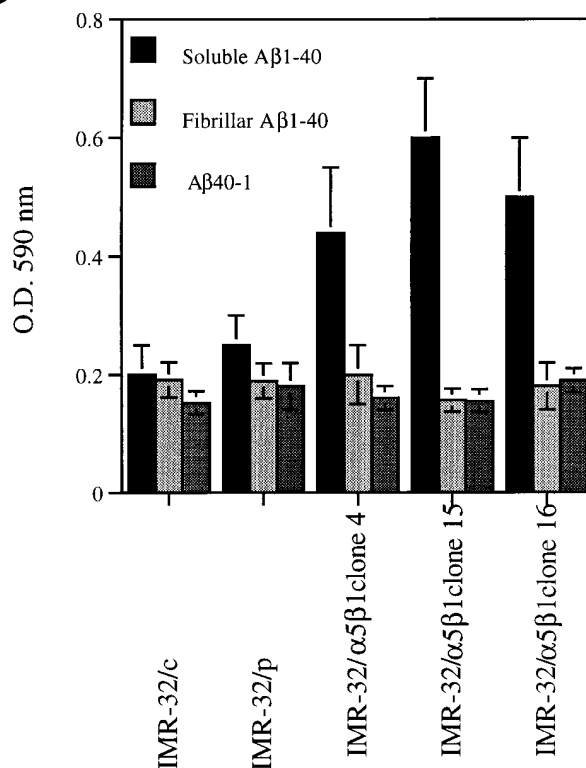


Figure 3. Adhesion of IMR-32 cells to coated A β . (A) Adhesion of IMR-32 cells transfected with the human $\alpha 5$ subunit IMR-32/ $\alpha 5 \beta 1$ and vector-transfected IMR-32/c cells to A β 1-40 was measured as described in the legend for Fig. 1. (B) IMR-32 cell transfectants and control IMR-32/c and IMR-32/p cells were plated on A β 1-40 coated in its soluble or fibrillar form or on the control peptide A β 40-1, and cell adhesion was measured as described in the legend for Fig. 1. Values in A and B represent the mean \pm SD; $n = 9$.

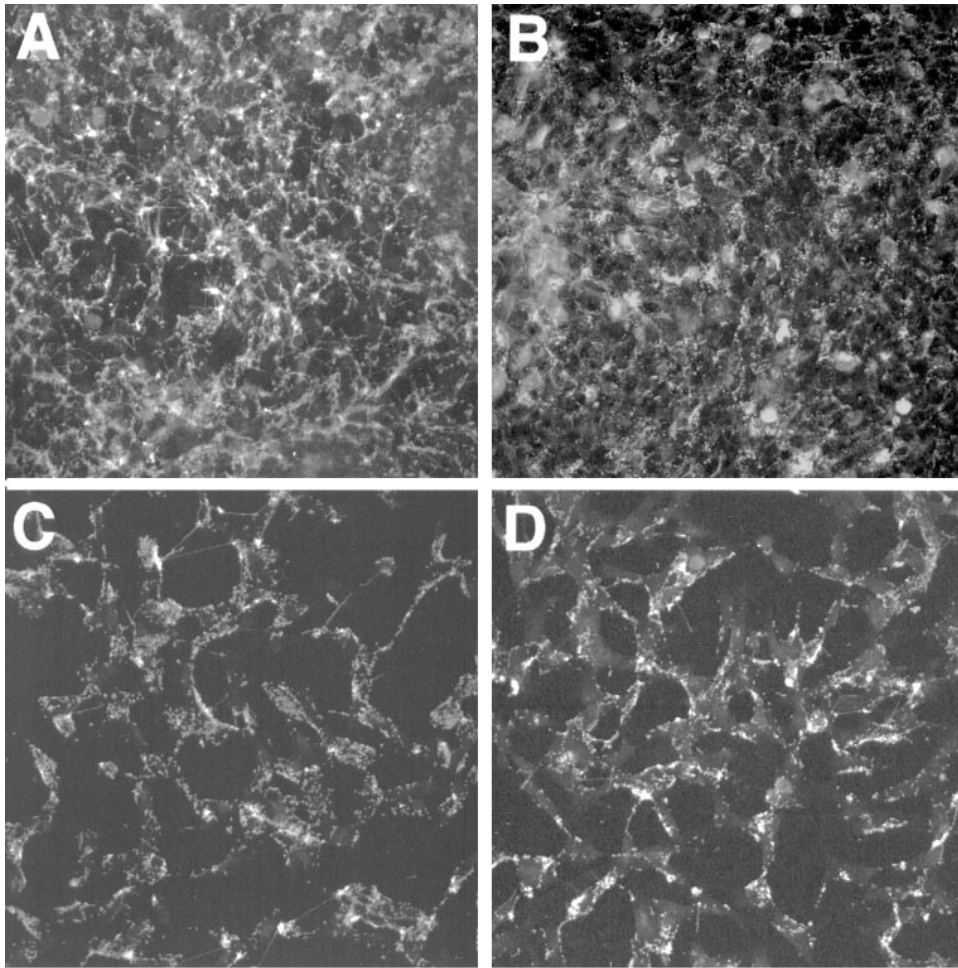


Figure 4. Immunofluorescent detection of reduced A β matrix in cultures expressing the $\alpha 5\beta 1$ integrin. Deposition of A β 1-40 matrix from soluble A β added to the culture media of IMR-32 control cells IMR-32/c (A) and IMR-32/p (B), and of $\alpha 5\beta 1$ expressing IMR-32 clones 4 (C) and 15 (D) was examined. The A β matrix was detected by a polyclonal anti-A β 1-40 antibody followed by a rhodamine-labeled secondary antibody. The experiment was repeated at least three times for each cell line, and representative results are shown. Bar, 50 μ m.

added to cell cultures formed a matrix around the cells that was detectable by immunostaining with anti-A β antibodies. There was a substantial decrease in the formation of matrix from added A β in cultures of the $\alpha 5\beta 1$ -expressing IMR-32 cell lines compared with the control lines (Fig. 4, A–D). Moreover, the matrix in the $\alpha 5\beta 1^+$ cell cultures appeared to be cell-associated, whereas in the $\alpha 5\beta 1^-$ cell cultures it appeared to be largely independent of the cells.

To study quantitatively the formation of the A β matrix, the various IMR-32 lines were incubated with 125 I-labeled A β for 72 h, and the amount of radiolabeled A β that had become soluble in detergent was measured. The IMR-32 clones expressing $\alpha 5\beta 1$ deposited fivefold less insoluble A β radioactivity than the control cells. Moreover, the P1D6 anti- $\alpha 5$ antibody returned A β matrix formation in the $\alpha 5\beta 1$ -expressing IMR-32 cultures to the level in the parental control cells (Fig. 5 A). A control antibody had no effect. CHO cells expressing $\alpha 5\beta 1$ also had less A β matrix than their control-transfected counterpart cells as judged from the insolubility of [125 I]A β ; the difference was fourfold (Fig. 5 B). Adding the anti- $\alpha 5$ antibody canceled the $\alpha 5\beta 1$ effect, but a control antibody did not. The insolubility of A β remained the same in the CHO control cell cultures regardless of the antibody added. These results indicate that cell expression of $\alpha 5\beta 1$ reduces A β matrix deposition threefold relative to the control cells. Because iodi-

nated A β forms fibrils less readily than unlabeled A β 1-40 (Bush et al., 1994), it was not possible to use the [125 I]A β to quantitate the proportion of the added A β 1-40 that becomes insolubilized.

Soluble A β 1-40 is Taken Up By Cells and Partially Degraded Via an $\alpha 5\beta 1$ -Mediated Pathway

Possible reasons for the $\alpha 5\beta 1$ -mediated reduction of A β matrix include internalization of soluble A β 1-40, degradation of the peptide, or both. Neuronal cells have been shown to internalize A β , but the mechanism for this internalization is only incompletely known (Ida et al., 1996, Hammad et al., 1997). To investigate the possibility that binding $\alpha 5\beta 1$ to soluble A β initiates cellular uptake of A β , we examined the processing of 125 I-labeled A β 1-40 by $\alpha 5\beta 1^+$ and $\alpha 5\beta 1^-$ cells. Initially, CHO-B2/c control cells and transfectants were incubated for 1 h with [125 I]A β 1-40, and were then examined for cell-associated radioactivity. The $\alpha 5\beta 1$ -expressing CHO-B2 cells contained twofold more radioactivity at 1 and 12 h than the control CHO-B2/c cells (Fig. 6 A).

Cell cultures were then incubated with 125 I-labeled A β over a 72-h period to determine whether the [125 I]A β taken up by the cells was degraded. $\alpha 5\beta 1$ -expressing IMR-32 cells contained twofold more radioactivity after the 72-h in-

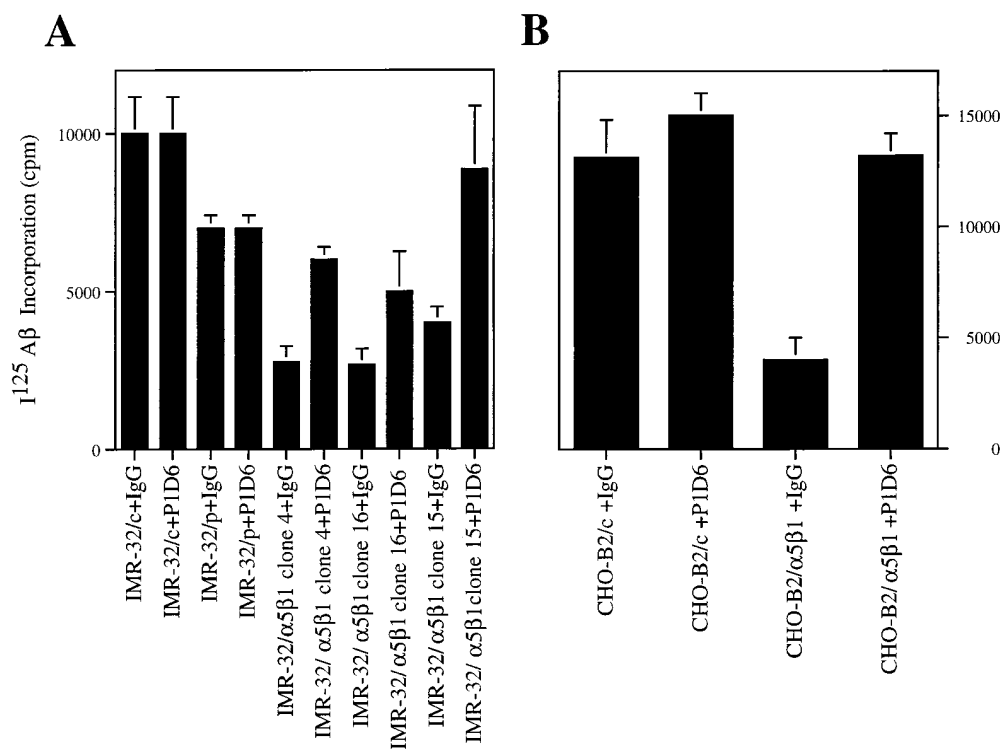


Figure 5. Quantitation of A β matrix deposition in cultures of IMR-32 cells and their α 5 β 1-expressing clones. (A) ¹²⁵I-labeled soluble A β 1-40 was incubated with cultures of two control lines and three α 5 β 1-expressing lines at 37°C for 72 h in the presence of either the monoclonal antibody to the human α 5 integrin subunit (P1D6) or control IgG. The total amount of ¹²⁵I-labeled A β 1-40 associated with an SDS soluble matrix was determined as described in Materials and Methods. The reduction of A β 1-40 matrix deposition in the α 5 β 1-expressing cultures was reversed by an anti- α 5 integrin monoclonal antibody (P1D6), but not by the control IgG. (B) CHO-B2/ α 5 β 1 cells show reduced deposition of [¹²⁵I] to A β matrix relative to control CHO cells. The experimental procedure was the same as in A. The

P1D6 antibody reversed the α 5 β 1-dependent matrix reduction, whereas the control mouse IgG had no effect. Experiments in A and B were repeated at least four times, and representative results are shown. Values represent the mean \pm SD; $n = 3$.

incubation than α 5 β 1-negative IMR-32 cells (Fig. 6 B). Part of the radioactivity was soluble in TCA, indicating that A β had been degraded. CHO cells internalized and degraded soluble A β in a similar manner, with α 5 β 1-expressing cells containing eightfold more TCA-soluble radioactivity than α 5 β 1-negative cells (Fig. 6 C). The CHO cells expressing α 5 β 1 bound 10% of the added A β , whereas the control cells bound only 0.4%. Moreover, 90% of the cell-associated A β was degraded in the CHO- α 5 β 1 expressers. The higher expression levels of α 5 β 1 on the CHO transfectants (Fig. 2, D and E) may explain why these cells bound and internalized more radiolabeled A β than the IMR-32 transfectants.

We next examined whether A β was released into the culture medium. The release of radioactivity into cell culture media was monitored over a 72-h period that followed a 1-h incubation with ¹²⁵I-labeled A β 1-40. The media of α 5 β 1-expressing IMR-32 and CHO cells contained twofold more radioactivity than the corresponding control cell media (Fig. 6, D and E). These results point to an α 5 β 1-dependent pathway that internalizes and degrades A β .

α 5 β 1 Protects Cells Against A β Induced Apoptosis

Having established an α 5 β 1-dependent mechanism for the inhibition of A β matrix deposition, we examined whether the reduction of the A β matrix would promote neuronal cell survival in cultures treated with A β . IMR-32 cell lines cultured with exogenous soluble A β 1-40 underwent apoptosis in the absence of α 5 β 1 (Fig. 7, A and B), but three α 5 β 1-expressing lines did not (two are shown in Fig. 7, C

and D). The control peptide A β 40-1 caused no apoptosis in the control (Fig. 7, E and F) or α 5 β 1-expressing cells (not shown). Analysis of acridine orange/ethidium bromide uptake revealed three times more apoptosis in the control cells than in the IMR-32 α 5 β 1-expressers (Fig. 8).

We also assessed the A β effect by using the MTT assay, which measures cell viability by detecting the ability of a mitochondrial enzyme to reduce its substrate. A β -treated IMR-32 control cells lost their ability to reduce MTT in a manner that was dependent on the dose of A β , whereas A β had almost no effect on the α 5 β 1-expressing cell lines (Fig. 9 A). The control peptide A β 40-1 had no effect on MTT reduction in any of the cell types, even at the highest test concentration (Fig. 9 B). To examine further the cytotoxicity of A β 1-40, we used an assay that measures the release of LDH upon cell lysis (Behl et al., 1994). A threefold increase in LDH levels relative to controls was seen in the α 5 β 1⁻ IMR-32 cells cultured in the presence of A β 1-40, whereas A β 1-40 had no effect on the LDH levels of the α 5 β 1⁺ cells (Fig. 10 A). These results indicate that α 5 β 1-mediated A β binding protects the IMR-32 cells from the cytotoxicity of aggregated A β , presumably by inhibiting its aggregation into fibrils. No apoptosis was caused by A β in any of the CHO cell lines, as examined by TUNEL staining, the MTT assay, and the LDH assay, indicating that these cells are resistant to the cytotoxic effects of an A β matrix.

We previously demonstrated that cell attachment through α 5 β 1 protects CHO cells from apoptosis when cultured in a serum-free environment (Zhang et al., 1995). Therefore, we examined whether ligation of α 5 β 1 to coated A β 1-40

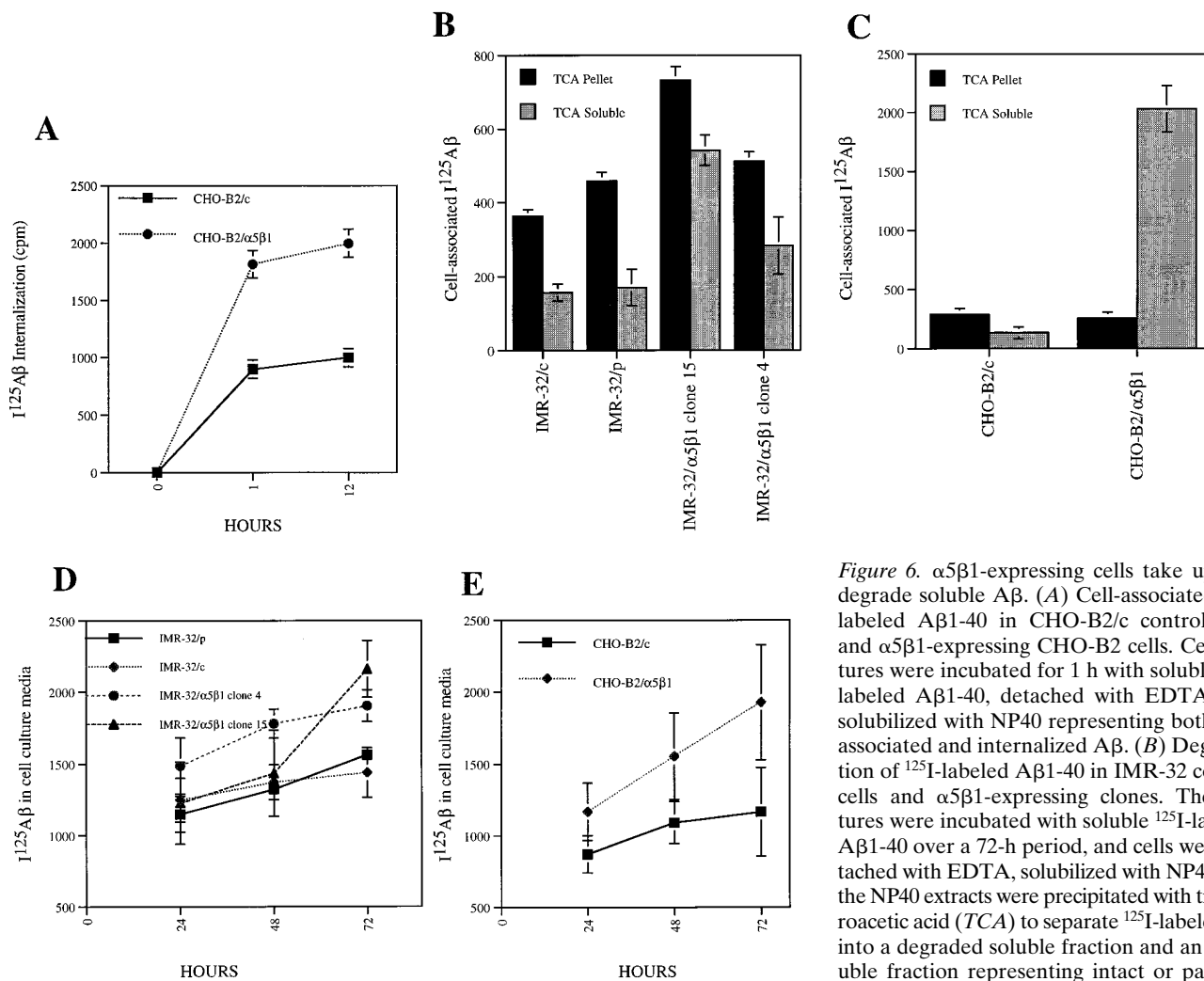


Figure 6. $\alpha 5\beta 1$ -expressing cells take up and degrade soluble A β . (A) Cell-associated ^{125}I -labeled A $\beta 1$ -40 in CHO-B2/c control cells and $\alpha 5\beta 1$ -expressing CHO-B2 cells. Cell cultures were incubated for 1 h with soluble ^{125}I -labeled A $\beta 1$ -40, detached with EDTA, and solubilized with NP40 representing both cell-associated and internalized A β . (B) Degradation of ^{125}I -labeled A $\beta 1$ -40 in IMR-32 control cells and $\alpha 5\beta 1$ -expressing clones. The cultures were incubated with soluble ^{125}I -labeled A $\beta 1$ -40 over a 72-h period, and cells were detached with EDTA, solubilized with NP40, and the NP40 extracts were precipitated with trichloroacetic acid (TCA) to separate ^{125}I -labeled A β into a degraded soluble fraction and an insoluble fraction representing intact or partially degraded A β . (C) Cell-associated ^{125}I -labeled

A $\beta 1$ -40 in CHO control cells (CHO-B2/c) and $\alpha 5\beta 1$ -expressing clones (CHO-B2/ $\alpha 5\beta 1$). (D and E) Release of [^{125}I] from ^{125}I -labeled A $\beta 1$ -40 into cell culture medium. ^{125}I -labeled soluble A $\beta 1$ -40 was added to the culture media of various IMR-32 (D) or CHO (E) cell lines and incubated for 1 h. After that, the media was removed, the cell layer was washed, and new media was added. The release of radioactivity into the media was monitored over a 72-h period. Experiments in A–E were repeated at least three times, and representative results are shown. Values in A–E represent the mean \pm SD; $n = 3$.

would protect $\alpha 5\beta 1$ -expressing CHO cells from apoptosis in serum-free cultures. CHO-B2/ $\alpha 5\beta 1^+$ cells were plated on either fibronectin, vitronectin, or A β -coated dishes and examined for survival 96 h after serum withdrawal. CHO-B2/ $\alpha 5\beta 1^+$ cells survived on A β and fibronectin, whereas cells plated on vitronectin underwent apoptosis (Fig. 10 B). These results indicate that $\alpha 5\beta 1$ can also protect cells from apoptosis by mediating cell attachment to coated A β .

Discussion

We report that the $\alpha 5\beta 1$ integrin mediates cell adhesion to A β and promotes internalization and degradation of A β . This $\alpha 5\beta 1$ -A β interaction correlates with both an increase in the clearance of soluble A β , a reduction in the formation of an insoluble A β fibrillar matrix, and a decrease of the toxicity of A β to cells. This study provides one mechanism for regulating A β accumulation.

Our data, showing that A β binds to $\alpha 5\beta 1$, and to a lesser extent $\alpha v\beta 1$, is in agreement with previous reports that A β mediates cell attachment, and that the RHD sequence in it serves as an integrin-binding site (Ghiso et al., 1992; Sabo et al., 1995). The RHD sequence apparently functions as a mimic of the RGD sequence in fibronectin, the matrix ligand of $\alpha 5\beta 1$ (Ruoslahti, 1996a), because a short peptide containing the RGD sequence inhibits A β binding. $\alpha 5\beta 1$ binds only to nonfibrillar A β , since we did not see any detectable cell adhesion to aggregated fibrillar A β . Therefore, other receptors presumably mediate cellular interactions with fibrillar A β , and are responsible for the cytotoxic effects of this form of A β . The $\alpha 5\beta 1$ integrin is one of the most discriminating of the RGD-directed integrins with regard to its ligand specificity (Ruoslahti, 1996b). In addition to its main ligand fibronectin, the $\alpha 5\beta 1$ integrin has only been shown to bind to the bacterial protein invasins (Watari et al., 1996) and the insulin-like growth factor binding protein IGFBP-X (Jones et al.,

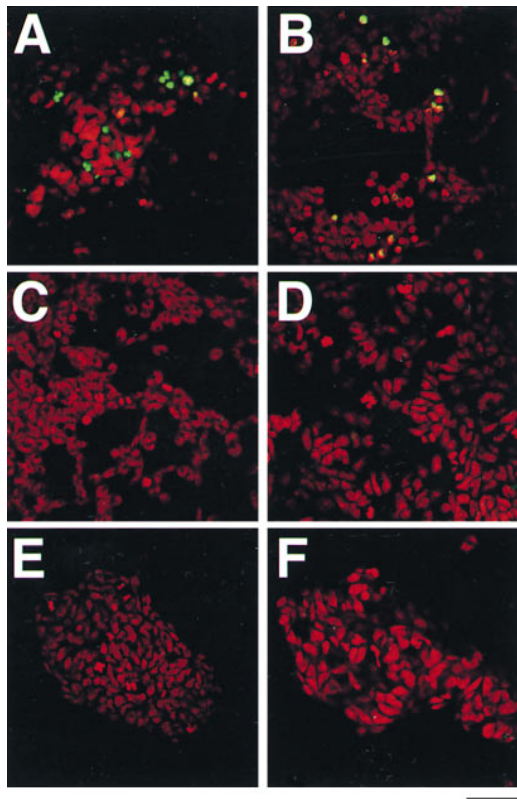


Figure 7. Effect of A β matrix on apoptosis. Expression of $\alpha 5\beta 1$ protects IMR-32 neuroblastoma cells from apoptosis induced by A β matrix formation. Cell cultures were incubated for 72 h with A $\beta 1$ -40 to allow aggregation of A β into a matrix, and the cultures were then examined for evidence of cytotoxicity by using the TUNEL assay. A percentage of IMR-32/c (A) and IMR-32/p (B) cells underwent apoptosis (green). The viable cells (red) are counterstained with propidium iodide. No apoptotic cells were seen in IMR-32/ $\alpha 5\beta 1$ transfectant clones 4 (C) and 15 (D) under the same conditions. (E–F) The IMR-32/c and IMR-32/p control cells remained viable when incubated for 72 h with the inactive control peptide A $\beta 40$ -1. These experiments were repeated at least three times for each cell line, and representative results are shown. Bar, 50 μ m.

1993). Our results add A β among its ligands. The binding site for $\alpha 5\beta 1$ seems to be available only in A β , not in its precursor protein (APP; B. Bossy, M.L. Matter, and E. Ruoslahti, unpublished results).

The $\alpha 5\beta 1$ integrin may play a role in the rapid clearance of A β that occurs in the normal brain (Gherzi-Egea et al., 1996). We show that expression of the $\alpha 5\beta 1$ integrin is associated with increased cellular uptake and degradation and decreased matrix deposition of A β in cell cultures. Moreover, reversal of this effect with a function-blocking anti- $\alpha 5$ antibody established a causal link between $\alpha 5\beta 1$ activity and increased clearance of A β . Although more complex explanations of this effect are possible, the binding of A β to $\alpha 5\beta 1$ shown here suggests that A β binds to $\alpha 5\beta 1$ at the cell surface, and is subsequently internalized into a cellular compartment where it is degraded. This hypothesis is in agreement with previous results showing that a neuronal cell line internalizes A β from culture medium in a manner that is dependent on the NH $_2$ terminus of A β

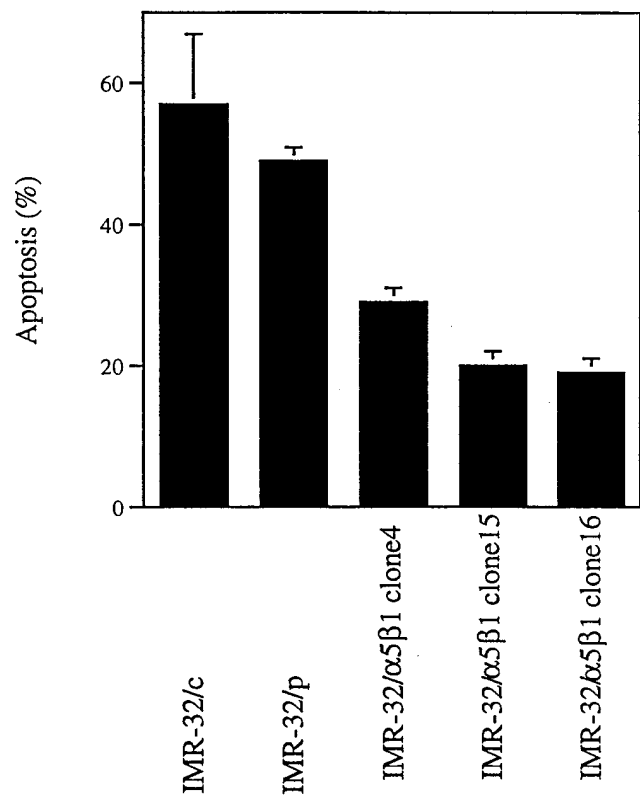


Figure 8. Effect of A β matrix on apoptosis. Expression of $\alpha 5\beta 1$ protects IMR-32 neuroblastoma cells from apoptosis induced by A β matrix formation. Cell cultures were incubated for 72 h with A $\beta 1$ -40 to allow aggregation of A β into a matrix, and the cultures were then examined for evidence of apoptosis by measuring the ability of cells to take up acridine orange/ethidium bromide. A higher percentage of $\alpha 5\beta 1^-$ IMR-32/c and IMR-32/p cells than $\alpha 5\beta 1^+$ transfectants underwent apoptosis. Values represent the mean \pm SD; $n = 9$.

where the RHD sequence resides (Ida et al., 1996). The lipoprotein Apo J can also reduce the formation of fibrillar A β by causing it to be internalized and degraded (Hammad et al., 1997). Thus, it is likely that more than one mechanism plays a role in the regulation of A β accumulation in vivo. Clearly, a transgenic animal expressing the amyloid precursor protein with a mutated RHD sequence would be of great interest in testing the contribution of the RHD sequence and integrin-binding to the metabolism of A β .

The $\alpha 5\beta 1$ integrin circulates through the endocytic cycle (Bretscher, 1989; Bretscher, 1992). Inhibiting exocytosis with primaquin causes accumulation of internalized $\alpha 5\beta 1$ in an intracellular pool that returns to the cell surface over time. Recent studies have shown that internalization of fibrillar A β promotes accumulation of stable fibrillar A β in the late endosome/secondary lysosome compartment, whereas internalization of soluble A β leads to degradation of the peptide in the same compartment (Knauer et al., 1992; Koo and Squazzo, 1994; Yang et al., 1995). This result is in agreement with our data, showing that soluble A β is internalized through an $\alpha 5\beta 1$ integrin-mediated pathway, and is at least partially degraded, presumably within endosomes. Thus, clearance of soluble A β can be mediated by the $\alpha 5\beta 1$ integrin, presumably through the receptor-

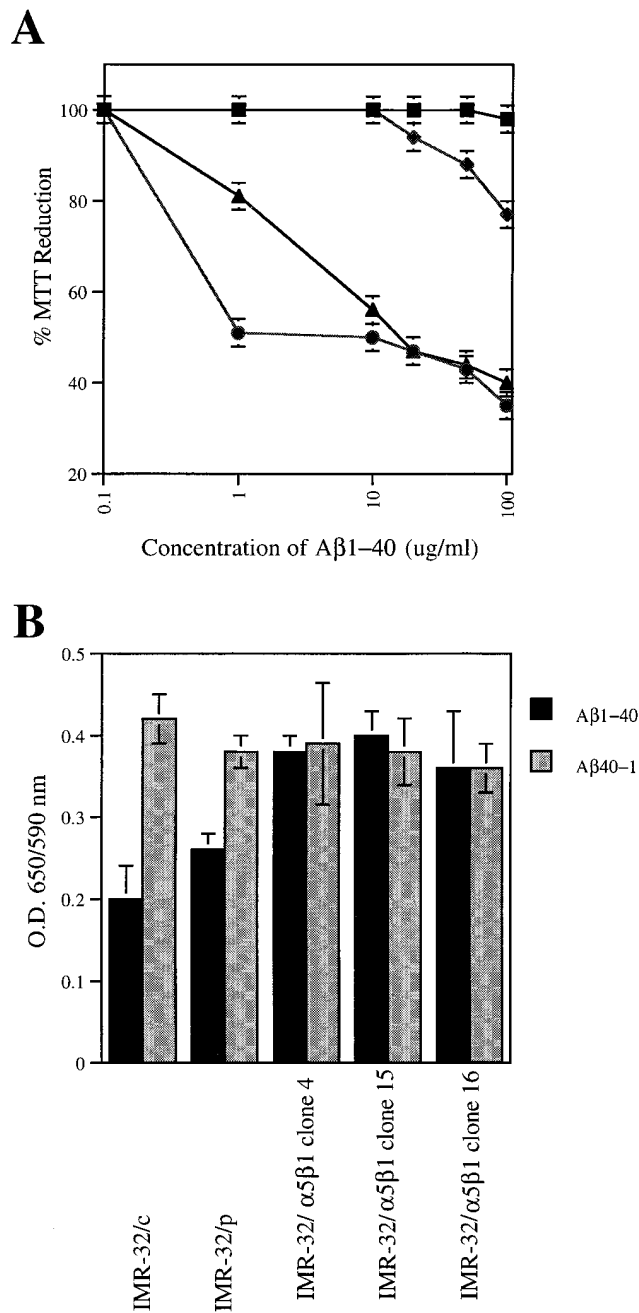


Figure 9. Effect of Aβ1-40 on cell viability. Cell survival in cultures containing aggregated Aβ. Cell cultures were incubated for 72 h with Aβ1-40 to allow aggregation of Aβ into a matrix, and the cultures were then examined for their ability to reduce MTT. (A) The control cells—IMR-32/c (circles) and IMR-32/p (triangles)—lost the ability to reduce MTT, whereas the IMR-32/α5β1 clones 4 (diamonds) and 15 (squares) were essentially resistant under the same conditions. Values represent the mean ± SD; *n* = 9. (B) The control peptide Aβ40-1 did not affect the ability of any cell type to reduce MTT.

mediated endocytosis pathway that normally internalizes this integrin.

α5β1 may play a protective role in the brain by suppressing Aβ cytotoxicity. We provide evidence for two separate mechanisms that could be responsible for such a protective effect. First, we show that α5β1-mediated adhe-

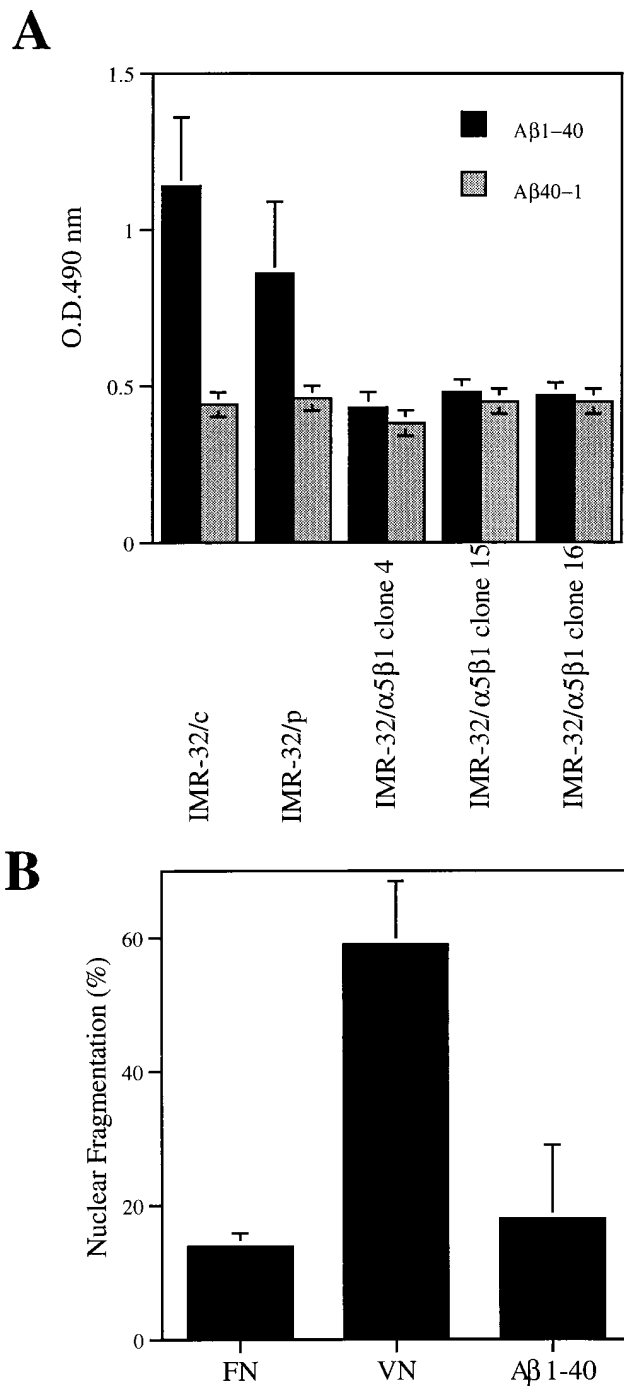


Figure 10. Cell survival in cultures containing aggregated Aβ. Cell cultures were incubated for 72 h with Aβ1-40 to allow aggregation of Aβ into a matrix, and the LDH levels within the cultures were analyzed. (A) The α5β1⁻ cells IMR-32/c and IMR-32/p showed increased LDH levels, whereas the IMR-32/α5β1 clones 4, 15, and 16 maintained LDH levels similar to those seen in the presence of the Aβ40-1 control peptide. Values represent the mean ± SD; *n* = 9. (B) Cell survival on Aβ coated from freshly made solution onto a plastic surface. Cells were seeded onto Aβ1-40, fibronectin, and vitronectin coated onto microtiter wells, cultured in serum-free conditions, and examined by DAPI staining after 96 h. The α5β1 transfectants remained viable on Aβ1-40 and fibronectin, but not on vitronectin.

sion to nonfibrillar A β protects cells from apoptosis in cell culture. Upregulation of Bcl-2 (Zhang et al., 1995) and activation of the MAPK pathway (Wary et al., 1997) may be responsible for this pathway. The second and potentially more important mechanism is suggested by our demonstration that α 5 β 1 suppresses the apoptotic effects of A β by reducing production of toxic A β matrix.

The α 5 β 1 integrin and α v β 1 are present in the adult central nervous system (Grooms et al., 1993). Immunostaining for α 5 β 1 shows that it is expressed in the vasculature, cortex, and hippocampus of adult rat brain (Bahr et al., 1991; Pagani et al., 1992; Tawil et al., 1994; for review see Sargent Jones, 1996). Moreover, primary hippocampal neurons express α 5 β 1 (Yamazaki et al., 1997). Soluble A β 1-40 is present in vivo (Seubert et al., 1992), and is rapidly cleared when injected into normal rats (Ghersis-Egea et al., 1996). Our results suggest that α 5 β 1 may mediate the clearance of A β , and that α 5 β 1 may play a significant role in protecting the brain from the A β -initiated pathology that in its extreme form causes AD.

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