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Syndecan-1 mediates the coupling of positively charged submicrometer amorphous silica particles with actin filaments across the alveolar epithelial cell membrane

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ABSTRACT

The cellular interactions and pathways of engineered submicro- and nano-scale particles dictate the cellular response and ultimately determine the level of toxicity or biocompatibility of the particles. Positive surface charge can increase particle internalization, and in some cases can also increase particle toxicity, but the underlying mechanisms are largely unknown. Here we identify the cellular interaction and pathway of positively charged submicrometer synthetic amorphous silica particles, which are used extensively in a wide range of industrial applications, and are explored for drug delivery and medical imaging and sensing. Using time lapse fluorescence imaging in living cells and other quantitative imaging approaches, it is found that heparan sulfate proteoglycans play a critical role in the attachment and internalization of the particles in alveolar type II epithelial cell line (C10), a potential target cell type bearing apical microvilli. Specifically, the transmembrane heparan sulfate proteoglycan, syndecan-1, is found to mediate the initial interactions of the particles at the cell surface, their coupling with actin filaments across the cell membrane, and their subsequent internalization via macropinocytosis. The observed interaction of syndecan molecules with the particle prior to their engagement with actin filaments suggests that the particles initiate their own internalization by facilitating the clustering of the molecules, which is required for the actin coupling and subsequent internalization of syndecan. Our observations identify a new role for syndecan-1 in mediating the cellular interactions and fate of positively charged submicrometer amorphous silica particles in the alveolar type II epithelial cell, a target cell for inhaled particles.

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Introduction

The potential toxicity or biocompatibility of submicrometer and nanoscale engineered particles is determined by the cellular pathways and interactions of the particles, which dictate the cellular response. The cellular interactions and subsequent response of the cells are governed by the physical and chemical properties of the particles (Balbus et al., 2007; Chithrani and Chan, 2007; Verma et al., 2008). Increase in positive surface charge has been shown to increase the cellular uptake of liposomes, polymeric and other particles for gene and drug delivery (Ewert et al., 2004; Lorenz et al., 2006; Basarkar et al., 2007). Increase in positive surface charge can also contribute to increased toxicity (Hoet et al., 2001; Fischer et al., 2003; Xia et al., 2006), but the molecules and mechanisms that mediate the charge-dependent cellular uptake or response are largely unknown.

Synthetic submicro- and nanoscale silica particles have been explored in drug delivery and medical imaging and sensing (Tallury et al., 2008; Kim et al., 2008; Moulari et al., 2008; Rigby et al., 2008). Synthetic amorphous silica particles have been also used in a wide array of industrial applications, such as the food or paint industry, creating a significant source for potential human exposure (Merget et al., 2002; Barik et al., 2008). Alveolar type II epithelial cells, which are distinguished by apical microvilli, are a potential target for these particles (Oberdorster et al., 2005; Donaldson et al., 2008). The cells play critical roles in the function of the alveoli (Weaver and Whitsett, 1991; Wikenheiser et al., 1993; Kasper and Haroske, 1996), and release chemokines when they are exposed to certain particles (Barrett et al., 1999; Calcabrini et al., 2004; Barlow et al., 2005), including amorphous silica (Hetland et al., 2001; Singal and Finkelstein, 2005), but the underlying cellular interactions of the particles that lead to the pro-inflammatory response are still unclear. Insights to the nature of these interactions has been recently gained by demonstrating charge-dependent coupling of positively charged submicro- and nanoscale amorphous silica particles with actin filaments across the membrane in these cells (Orr et al., 2007; Hess and Tseng, 2007). The observations suggest that the interactions of the particles at the cell surface are mediated by a negatively charged transmembrane molecule that interacts either directly or indirectly with the actin filaments.

One of the most negatively charged family of molecules at the cell surface is the sulfated proteoglycans family. Sulfated proteoglycans

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consist of a core protein that is covalently linked to one or two types of glycosaminoglycans, which are long linear polysaccharide chains that bear negative charges. The sulfated proteoglycans are a highly diverse family of molecules that are abundant in the extracellular matrix of all tissues, and some that are expressed at the cell surface. However, only a few sulfated proteoglycans are transmembrane molecules that potentially could couple the particles with actin filaments across the cell membrane. Among them are the syndecans, a sub-family of four transmembrane proteoglycans (Carey, 1997; Tkachenko et al., 2005) that carry heparan sulfate or chondroitin sulfate chains on their extracellular domains (Rapraeger et al., 1985; Shworak et al., 1994; Deepa et al., 2004). The four syndecans have an identical PDZ binding motif at their cytoplasmic C-terminus (Tkachenko et al., 2005) that allows them to bind PDZ proteins, such as syntenin (Grootjans et al., 1997; Grootjans et al., 2000; Zimmermann et al., 2001) or CASK (Cohen et al., 1998; Hsueh et al., 1998). Syntenin and CASK, in turn, bind actin via actin binding proteins, such as merlin (Kang et al., 2003; Scoles, 2008) or protein 4.1 (Cohen et al., 1998), respectively.

The association of syndecans with actin requires their clustering or cross linking (Rapraeger et al., 1986; Carey et al., 1994; Carey et al., 1996). Syndecans bind extracellular matrix molecules and play important roles in the regulation of focal adhesion, actin bundling, lamellipodia spreading and cell migration (Longley et al., 1999; Yoneda and Couchman, 2003; Chakravarti et al., 2005; Tkachenko et al., 2006). The binding of syndecans to their matrix ligands leads to their clustering and association with actin that, in turn, could anchor the cytoskeleton to the extracellular matrix (Rapraeger et al., 1986; Bernfield and Sanderson, 1990).

The clustering of syndecans is also required for their internalization (Fuki et al., 1997; Fuki et al., 2000). Syndecans can act as growth factor receptors and mediate their internalization and signaling (Tkachenko et al., 2005). Syndecan-4, for example, mediates the internalization and signaling of fibroblast growth factor 2 through the activation of Rac1 and internalization via macropinocytosis (Horowitz et al., 2002; Bass and Humphries, 2002; Tkachenko et al., 2004), a fluid phase endocytic process that is associated with a dynamic rearrangement of actin filaments and the induction of membrane ruffling in response to receptor activation or other external stimuli (Conner and Schmid, 2003). Heparan sulfate proteoglycans mediate the internalization of certain cationic–DNA complexes that are designed for gene delivery (Mislick and Baldeschwieler, 1996; Mounkes et al., 1998), or polycationic macromolecules and peptides that are designed to deliver cargo proteins and molecules into cells (Poon and Gariépy, 2007). The internalization of certain cationic peptides also occurs via macropinocytosis following their binding to heparan sulfate proteoglycans (Nakase et al., 2007).

Here we show that heparan sulfate proteoglycans play a critical role in the attachment and internalization of positively charged submicrometer precipitated amorphous silica particles in alveolar type II epithelial cells, and that chondroitin sulfate chains have a significant but smaller role in this process. Specifically, syndecan-1, which is expressed predominantly by epithelial cells and carries heparan sulfate as well as chondroitin sulfate chains, is found to mediate the initial interactions of the particles at the cell surface, their coupling with actin filaments across the cell membrane, and their internalization pathway. The cellular fate of the particles is therefore determined by syndecan-1.

Materials and methods

Cell-culture. The alveolar type II epithelial cell line, C10, was used in these studies. C10 is a non-tumorigenic cell line that was derived from normal adult mouse lung and has been shown to preserve its characteristic phenotype (Malkinson et al., 1997). The cells were grown in CMRL 1066 growth medium, supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 g/ml streptomycin, all from Invitrogen (Carlsbad, CA). Cells were grown in 35 mm glass cover-slip bottom plates (WPI Sarasota, FL), and imaged in either physiological buffer (162 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl2, 10 mM glucose, 10 mM HEPES, pH 7.4) containing 2% BSA (Fisher), or in their growth medium.

Fluorescence probes and drug treatments. Syndecan-1 was labeled using a goat anti mouse antibody specific to the extracellular domain (aa 18–252) of the core protein (R&D Systems, Minneapolis, MN). The antibody was tagged either directly with Alexa 488 or Alexa 647, or with a rabbit anti goat antibody tagged with one of the two dyes (Molecular Probes/Invitrogen). Cells were incubated in the physiological buffer, containing 2% BSA for 30 min before incubation with the antibody for 10 min, all in room temperature. The cells were washed and imaged alive. Particle co-localization with 70 kDa dextran-rhodamine B (Molecular Probes/Invitrogen) was used to determine internalization via macropinocytosis. Cells were incubated with dextran-rhodamine B (1 mg/ml) together with the particles, tagged with Alexa-488, in buffer for 2 h at room temperature, washed and incubated in growth medium for additional 4 h in 37° before imaging. 5-(N-ethyl-N-isopropyl) amiloride (Sigma, St. Louis, MO) was used to block macropinocytosis. Cells were incubated with 1.5 mM amiloride in their normal growth medium for 15 min in 37° before adding the particles and during the following 4 h incubation before imaging. For this experiment, the cells were also incubated with wheat germ agglutinin — Alexa 647 (WGA-Alexa 647) (Molecular Probes/Invitrogen) at 20 g/ml to tag the plasma membrane. WGA-Alexa 647 and the particles, tagged with Alexa-546, were incubated as described above to identify internalized particles within membrane vesicles.

Particle characterization. Positively charged particles were achieved by modifying their surface with amine groups. 500 nm precipitated amorphous silica particles were obtained commercially, either as unmodified bare particles (Polysciences, Inc) that were modified as described below, or as aminated particles (Corpuscular, Inc., Cold Spring, NY). Using Zeta potentiometry, the unmodified particles were found to be negatively charged, having a zeta potential of negative 40 mV at pH 7.4. Our aminated particles were found to have a zeta potential of positive 20 mV at pH 7.4. The solution precipitation methods that are used to synthesize these particles occur at ambient or relatively low temperatures, resulting in the formation of amorphous silica particles. The formation of amorphous particles was confirmed for our particles by X-ray and electron diffraction. The colloidal nature of the particles was confirmed using transmission electron microscopy (TEM) (Fig. 1),

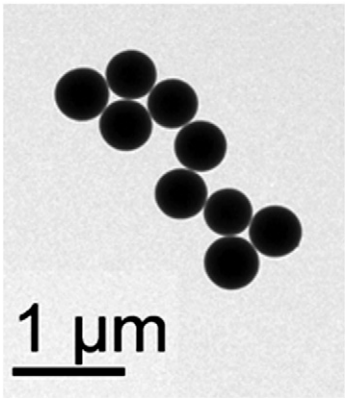

Fig. 1. TEM image of 500 nm precipitated amorphous silica particles that are used in this study.

and the size distribution was determined from the TEM images showing an average of 462 ± 20.5 nm in diameter. The dispersed nature of the particles in the experimental medium is demonstrated in Fig. 2A, where the majority of the particles are found within the first population, indicating their presence as individual particles.

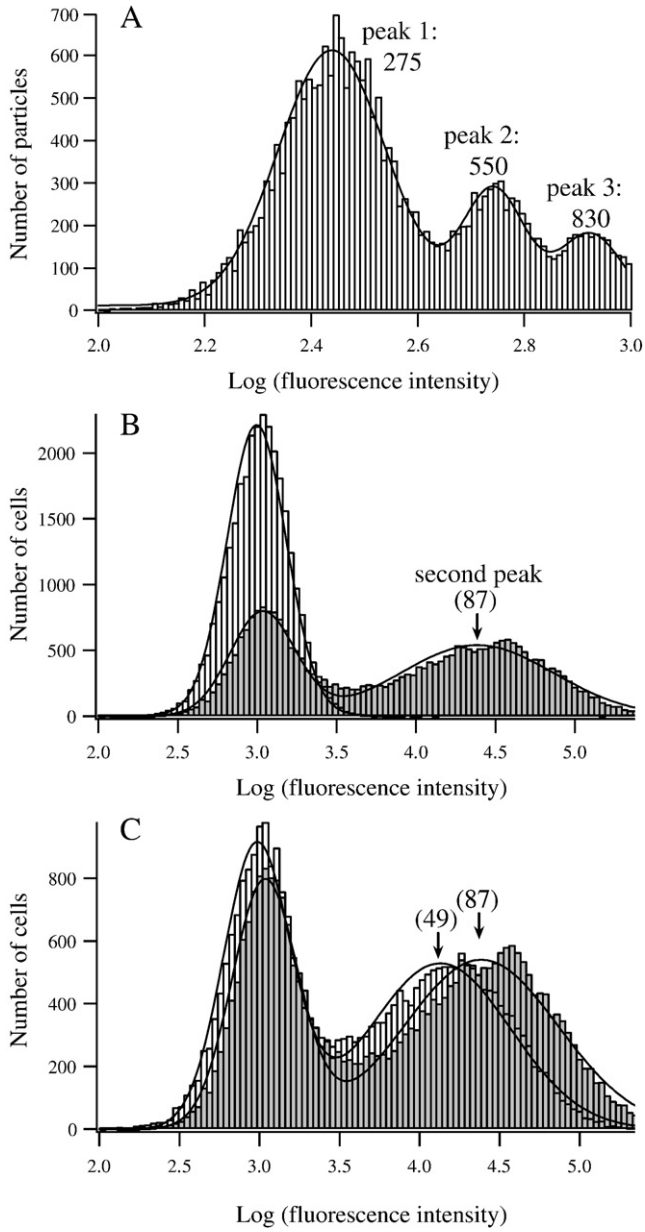

Fig. 2. Alveolar type II epithelial cells robustly capture and internalize positively charged 500 nm amorphous silica particles. (A) The flowcytometry histogram of fluorescent particles shows three distinct populations that indicate the presence of mostly individual particles (first population), as well as clusters containing two (second population) and three particles (third population). The average fluorescence intensity of individual particles is 275 counts, as determined from the incremental increase in the intensity values at the peaks. (B) The fluorescence intensity histogram of control cells, which are not exposed to the particles (open bars), peaks at low fluorescence intensity that results from the cellular auto-fluorescence. The fluorescence intensity histogram of cells that are incubated with the particles (filled bars) shows two distinct cell populations, where the second population consists of cells that are associated with the fluorescent particles. Using the fluorescence intensity of individual particles it is found that individual cells become associated, on average, with 87 particles per cell. (C) Using trypan blue to distinguish between particles at the cell surface and those that are internalized it is found that from the average of 87 particles that are associated with the cell (filled bare — before incubation with trypan blue), 49 particles are found, on average, inside the cell (open bars — after incubation with trypan blue).

Particles were sonicated by placing the tube with the particle solution in a bath sonicator before being applied to the cells. Our experimental approach allowed the identification of individual particles, either by DIC or fluorescence microscopy. Particles were added to the cells at concentrations that were adjusted experimentally to give an exposure in the order of 10–300 particles per cell, as estimated by DIC and fluorescence imaging (0.5–50 g/ml particles, in 1 ml final volume over a 35 mm plate, carrying cells at 70%–90% confluence). Identical concentrations were added when comparing between treatments.

Surface amination and fluorescence tagging of the particles. Particles were aminated either commercially or in our laboratory with (3-aminopropyl) triethoxysilane (APTES). 500 nm bare particles were reacted with APTES (Sigma-Aldrich, St. Louis, MO) in toluene at 80°C for 6 h. APTES solution amounts were 1.5 times the amount that would give monolayer coverage. After reaction, the particles were separated by centrifugation and washed repeatedly in toluene and ethanol. The particles were tagged with a fluorescent dye by reacting the succinimidyl ester conjugated Alexa 546 or Alexa 488 (Molecular Probes/Invitrogen) directly with amine groups on the particles. The particles were placed into dimethylformamide (DMF) (Sigma-Aldrich) at 25 mg/ml, were dispersed by ultrasonication, and various amounts of the dye in DMF were added to the suspensions. Reaction occurred at room temperature for 2–3 h under rotation. After reaction, the particles were separated by centrifugation and repeatedly washed in DMF, ethanol, and water. The dye concentrations were controlled to achieve lower coverage of dye molecules per particle for microscopy (5–10 dye molecules per particle), and higher coverage for flowcytometry (20–30 dye molecules per particle). The expected fluorescence intensity of a single fluorescent dye molecule, under our specific experimental conditions, was determined by the peak of the fluorescence intensity distribution that was generated by imaging hundreds of individual dye molecules spin-coated at pM concentration on a glass slide. This was done using single-molecule fluorescence detection methods, as described earlier (Orr et al., 2005; Orr et al., 2007). Similar fluorescence intensity histograms were generated for the fluorescence-tagged particles, and the ratio between the peak distributions of the particle and the dye gave the estimated number of dye molecules per particle.

Flowcytometry and enzyme treatments. FACSAria and the FACSDiva acquisition software (BD Biosciences, San Jose, CA) were used. Cells, grown to 80%–90% confluence, were exposed to the particles, tagged with Alexa 488, for 3 h. The cells were washed, dissociated off the plates using trypsin, and span down and resuspended in PBS. To cleave heparan sulfate or chondroitin sulfate chains, the cells were incubated with 5 U of heparinase I and II or chondroitinase ABC (both from Sigma) for 2 h prior to incubation with the particles. The green laser was used to excite the particles, and 30,000 cells per treatment were screened to assess the degree of particle association and internalization under each treatment. The raw data were transferred, using R (Free software) or Flowjo (Tree Star, Ashland, OR) to Igor Pro 6 (WaveMetrics, Portland, OR) for further analysis. Histograms were generated, overlaid and fitted with bimodal and trimodal Gaussian distributions for extracting fluorescence intensity values at the peak of the distributions.

Time lapse fluorescence and DIC imaging. Total internal reflection (TIRF) fluorescence laser microscope (Axiovert 200, Zeiss), equipped with a 100× oil-immersion objective (Plan-Apochromat, N.A. = 1.4, Zeiss) and a 2× relay lens in the emission path to the CCD camera was used. The overall magnification was 200×, leading to 100 nm per image pixel. A blue laser (Innova, Coherent) was used to excite syndecan-1 (Alexa 488) at 488 nm, a green laser (Nd:YAG Verdi V-10,

Coherent) was used to excite the particles (Alexa 546) at 532 nm, and a red laser (diode pumped solid state lasers, Crystalaser, Reno, NV) was used to excite WGA Alexa-647 at 632 nm. The lasers were coupled by a fiber coupler to the TIRF module, and shutters, controlled by the CCD controller, were set in front of the laser beams. Dichroic mirrors (Chroma Technology) were set to split the emissions of Alexa 488 and Alexa 546 at 550 nm, and to split the emissions of Alexa 546 and Alexa 647 at 600 nm. Fluorescence images were acquired by a back-illuminated nitrogen-cooled CCD camera (Spec-10 1340×700, Roper Scientific) with 90% quantum efficiency and single-molecule sensitivity. It is estimated that one intensity count in the CCD image corresponds to 2 detected photons. DIC images were taken at 200× magnification, using the above CCD camera. Images where acquired simultaneously at different regions on the CCD chip and were overlaid with 1 pixel (100 nm) registration accuracy using MATLAB (The MathWorks).

Laser scanning confocal microscopy. Olympus Fluoview FV300 was used. The microscope was coupled to two, 542 nm and 632 nm diode pumped solid state lasers (Crystalaser, Reno, NV), and a 488 nm air-cooled laser (Melles Griot, Carlsbad, CA), for exciting Alex 546 or rhodmine B, Alexa 647, and Alexa 488, respectively. A100× magnification oil emersion objective was used to scan an area of 1024 by 1024 pixels, leading to 140 nm per image pixel. Image analysis was done using the analysis tools in Volocity (Improvision, Waltham, MA), while zooming onto individual particles and toggling between the channels to determine co-localization, pixel by pixel.

Results

Quantifying particle attachment and internalization using flowcytometry

To determine whether proteoglycans carrying heparan sulfate and/or chondroitin sulfate chains play a role in the attachment and subsequent internalization of positively charged sub-micrometer engineered particles we used flowcytometry. This approach enables quantifying the association and internalization of the particles, while modifying the heparan sulfate or chondroitin sulfate chains at the cell surface. Alveolar type II epithelial cells (C10) were exposed to 500 nm precipitated amorphous silica particles, positively charged by amine groups and tagged with a fluorescent dye. Fig. 2 describes the quantitative approach that we took for calculating the average number of particles that become associated with an individual cell, and the fraction of the associated particles that is internalized by the cell. Using flowcytometry to screen thousands of particles, tagged with a fluorescent dye (Alexa 488), it was possible to identify the fluorescence intensity of individual particles. The flowcytometry histogram shows three distinct populations that peak at increments of about 275 fluorescence intensity counts (Fig. 2A). The incremental peaks indicate that in addition to individual particles, the solution contained smaller populations of particle clusters, containing two or three particles per cluster. This distribution indicates that the fluorescence intensity of individual particles is 275 counts. Control cells that are not exposed to the particles show a tight distribution in

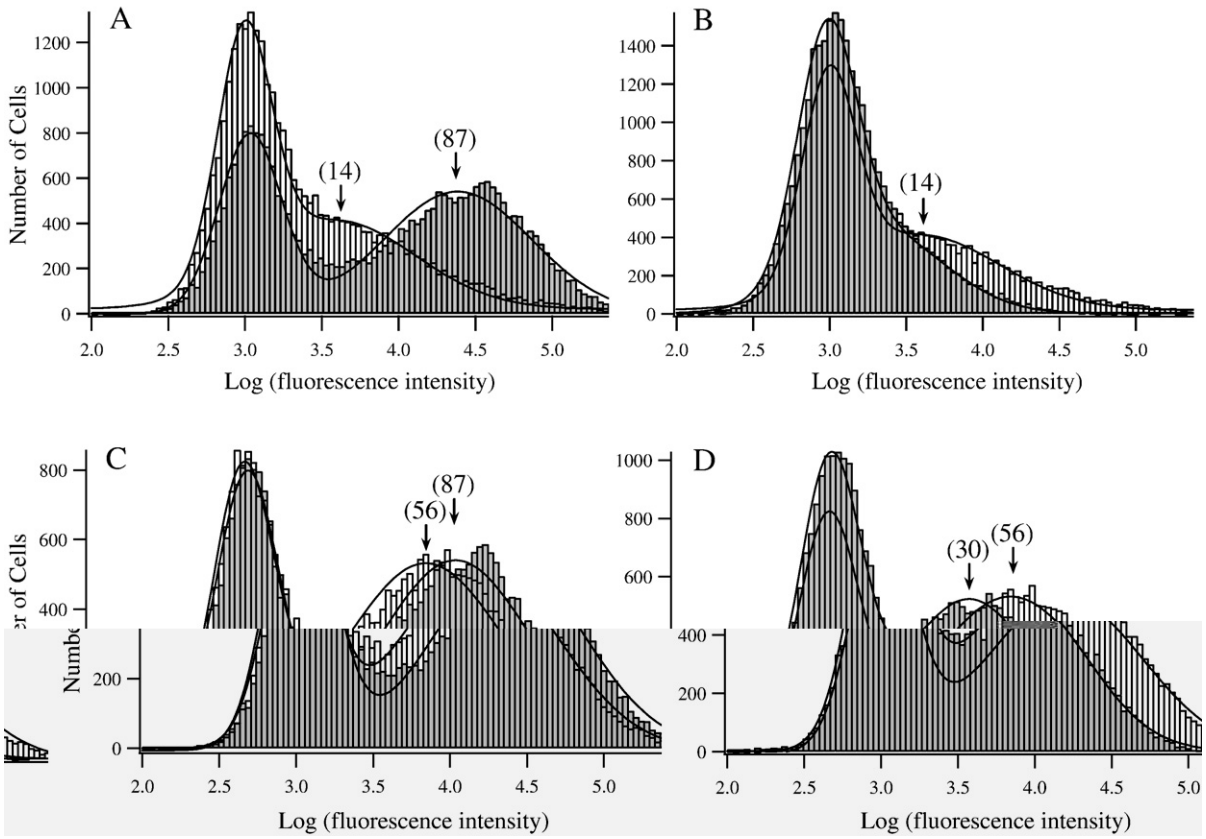

Fig. 3. Proteoglycans carrying heparan sulfate chains play a critical role in the attachment and internalization of positively charged 500 nm amorphous silica particles, while chondroitin sulfate chains play a smaller yet significant role in this process. (A) Treating the cells with heparinase leads to a dramatic decrease in the degree of particle association with the cell. From an average of 87 particles per untreated cell (filled bars), heparinase treatment reduces particle association to an average of 14 particles per cell (open bars). (B) Incubating heparinase-treated cells with trypan blue to quench the fluorescence particles at the cell surface leads to a near complete disappearance of the second peak (filled bars), indicating minimal particle internalization. (C) Treating the cells with chondroitinase leads to a smaller but significant decrease in the degree of particle association with the cell. From an average of 87 particles per untreated cell (filled bars), chondroitinase treatment (open bars) reduces particles association to an average of 56 particles per cell. (D) Incubating chondroitinase treated cells with trypan blue shows that about half of the associated particles are internalized. From the average of 56 associated particles per cell (open bars), an average of 30 particles per cell is found in the cytoplasm.