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Cell-Surface Nucleolin is a Signal Transducing P-Selectin Binding Protein for Human Colon Carcinoma Cells

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Abstract

We have previously shown that P-selectin binding to Colo-320 human colon carcinoma cells induces specific activation of the $\alpha_5\beta_1$ integrin with a concomitant increase of cell adhesion and spreading on fibronectin substrates in a phosphatidylinositol 3-kinase (PI 3-K) and p38 MAPK-dependent manner. Here, we identified by affinity chromatography and characterized nucleolin as a P-selectin receptor on Colo-320 cells. Nucleolin mAb D3 significantly decreases the Colo-320 cell adhesion to immobilized P-selectin-IgG-Fc. Moreover, nucleolin becomes clustered at the external side of the plasma membrane of living, intact cells when bound to cross-linked P-selectin-IgG-Fc chimeric protein. We have also found P-selectin binding to Colo-320 cells induces tyrosine phosphorylation specifically of cell-surface nucleolin and formation of a signaling complex containing cell surface nucleolin, PI 3-K, and p38 MAPK. Using siRNA approaches, we have found that both P-selectin binding to Colo-320 cells and formation of the P-selectin-mediated p38 MAPK/ PI 3-K signaling complex require nucleolin expression. These results show that nucleolin (or a nucleolin-like protein) is a signaling receptor for P-selectin on Colo-320 cells and suggest a mechanism for linkage of nucleolin to P-selectin-induced signal transduction pathways that regulate the adhesion and the spreading of Colo-320 on fibronectin substrates.

Keywords

P-Selectin; cell-surface nucleolin; cell adhesion; signal transduction

Introduction

Metastasis is a multi-step cascade of cellular events by which cancer cells leave the original tumor mass and establish new colonies at distant sites in the body [1,2]. In the hematogenous phase of metastasis, cancer cells that reach the blood stream must eventually gain the ability to attach to the blood or lymphatic vessel endothelium prior to successful tumor cell transendothelial migration and colonization of host organs [3,4]. Both integrins and selectins are thought to be involved in the process of tumor cell metastasis.

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Selectins are vascular adhesion molecules involved in leukocyte trafficking, inflammation, thrombosis, autoimmunity, and cancer. Three major members of the selectin family have been identified: L-selectin, E-selectin and P-selectin. L-Selectin is constitutively expressed on leukocytes. P- and E-selectins are expressed on activated endothelial cells. P-selectin is also expressed on thrombin-activated platelets. All three members of the selectin family can bind to human tumor cells and cancer-derived cell lines [5-11].

Both E-selectin and P-selectin can support the tethering of tumor cells to endothelium in flow conditions [6,12,13], and P-selectin can induce interactions between cancer cells and platelets to form emboli which facilitate the arrest of cancer cells [7,13-17]. Moreover, P- and/or L-selectin-deficient mice reveal a pronounced inhibition of metastasis compared with wild-type mice [18,19]. The absence of P-selectin also reduces primary tumor growth compared to P-selectin wild-type controls [20].

Integrins are transmembrane glycoproteins that function as cell adhesion receptors for cell-substrate and cell-cell interactions. Each integrin consists of an α and a β subunit. Integrin-mediated cell adhesion is subject to multiple signaling pathways that can control either the affinity of integrins for their ligands or the clustering of integrins on the cell surface [21-23]. It is believed that dysregulation of integrin-mediated cell adhesion can contribute to the promotion of the metastatic process [24-26].

Recently, we have shown that P-selectin binding to Colo-320 human colon carcinoma cells specifically activates the $\alpha 5 \beta 1$ integrin, which results in the increase of cell attachment and cell spreading on fibronectin substrates. This selectin-mediated integrin activation results from at least two distinct intracellular signaling pathways—the p38 MAPK and the PI 3-K pathways—that are linked by a p38 MAPK-PI 3-K signaling complex [27]. These results suggest that P-selectin binding to specific receptors on cancer cells can activate signals that regulate tumor cell adhesion and possibly proliferation.

Here, we have identified nucleolin (or a nucleolin-like protein) as a Colo-320 cell P-selectin receptor by using affinity chromatography, mass-spectrometry (MS), immunoassays, and RNAi knock-down. We show that nucleolin is expressed on the cell-surface. An anti-nucleolin mAb (D3) significantly blocks Colo-320 cell adhesion to immobilized P-selectin. Moreover, nucleolin becomes clustered on the plasma membrane when bound P-selectin-IgG-Fc chimeric protein is cross-linked. We also found that P-selectin binding to Colo-320 cells induces tyrosine phosphorylation specifically of cell-surface nucleolin, but not of nucleolin expressed in cytoplasm or nucleus, and formation of a nucleolin/p38 MAPK/PI 3-K signaling complex. Using siRNA to inhibit specifically nucleolin expression in Colo-320 cells, we have demonstrated that both the P-selectin binding to Colo-320 cells and formation of the P-selectin-mediated p38 MAPK/PI 3-K signaling complex require nucleolin expression. These results show that nucleolin is a signaling P-selectin receptor on Colo-320 human colon carcinoma cells and suggest a mechanism for linkage of nucleolin to P-selectin-induced signal transduction pathways that regulate cell adhesion through the activation of the $\alpha 5 \beta 1$ integrin.

Experimental Procedures

Materials

Recombinant murine P-selectin-IgG-Fc fusion protein was expressed in COS cells and purified as described [28] by the NIEHS Protein Expression Core Facility. The expression vector for the P-selectin-IgG-Fc fusion protein was a generous gift from Dr. John Lowe, University of Michigan Medical Center. Recombinant murine E- and L-selectin-IgG-Fc fusion proteins were obtained from R&D systems, Inc (Minneapolis, MN). Unlabeled control mouse IgG and human IgG-Fc were obtained from Accurate Chemical Co. (Westbury, NY). Anti-P-selectin

glycoprotein ligand 1 (PSGL-1) and anti-CD24 mAbs were obtained from Immunotech (Westbrook, ME). Anti-phosphotyrosine mAb (4G10) and anti-p38 MAPK (SAPK2 α /p38) antibodies were from Upstate Cell Signaling Solutions (Billerica, MA). Antibodies to phospho-p38 MAPK and PI 3-K p85 were from Promega (Madison, WI) and Cell Signaling (Danvers, MA), respectively. The anti-PI 3-K p110 β , anti-Na⁺/K ATPase α 1, and anti-histone 3 rabbit polyclonal antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA). The anti-nucleolin mAb (D3) was a generous gift from Dr. Jau-Shyong Deng, University of Pittsburgh School of Medicine. The other anti-nucleolin monoclonal and rabbit polyclonal antibodies were obtained from Stressgen (4E2) and Santa Cruz Biotech (H-250 and MS-3). R-Phycoerythrin (PE)-conjugated AffiniPure F(ab')₂ fragment of goat Fc-specific anti-human IgG was from Immunotech, and Alexa^{TM} 488- and Alexa^{TM} 564-coupled goat anti-mouse IgG and anti-rabbit IgG were obtained from Molecular Probes (Eugene, OR). Gelatin and Triton X-100 were obtained from Sigma (Saint Louis, MO). Paraformaldehyde was obtained from Electron Microscopy Sciences (Hatfield, PA). Goat serum was obtained from Zymed Laboratories, Inc. (South San Francisco, CA). Dimethylsulfoxide (DMSO) was obtained from the American Type Culture Collection (Manassas, VA).

Cell culture and preparation

Colo-320 HSR human colon adenocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured as described [27]. Cell lysates for biochemical analyses and flow cytometric analyses were described previously [27]. All protein concentrations were determined using the BCA assay (Pierce, Rockford, IL).

Cell fractionation

Cell fractionation was carried out using a modification of a previously published procedure [29]. Briefly, Colo-320 cells were harvested, frozen in a dry ice/methanol bath, and then resuspended in ice-cold hypotonic buffer 1 (10 mM Hepes, pH 7.5, 10 mM KCl and 1 mM dithiothreitol (DTT), 1.5 mM MgCl₂) containing 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin A. After incubating for 10 min on ice, the nuclei were sedimented by centrifugation at 500 $\times$ g for 10 min at 4°C and saved. The supernatant was then centrifuged twice at 800 $\times$ g for 10 min at 4°C, and the pellet was discarded. The supernatant was then centrifuged at 100,000 $\times$ g for 60 min at 4°C to pellet the cell membranes, and supernatant recovered as cytoplasmic fraction. Membrane and nuclear fraction pellets were washed twice with buffer 1. The nuclear and cell membrane fractions were separately solubilized in 150 mM NaCl, 2 mM EDTA, 50 mM Hepes, 0.5% deoxycholic acid, 1% Nonidet P-40, pH 7.5 containing 2 mM sodium orthovanadate, 1 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, 15 μ g/ml aprotinin, 15 μ g/ml leupeptin, and 15 μ g/ml pepstatin A for 60 min at 4°C and then cleared by centrifugation at 16,000 $\times$ g for 10 min at 4°C.

Biotinylation and purification of cell-surface proteins

Cells (3×10^8) were harvested, washed twice with ice-cold PBS with 1 mM CaCl₂ and 1 mM MgCl₂ (PBS⁺), and resuspended in 50 ml of a freshly prepared solution of 0.5 mg/ml sulphosuccinimidobiotin (sulfo-NHS-biotin, Pierce, Rockford, IL,) in PBS⁺. After incubation for 30 min at 4°C, the biotinylation reaction was quenched by adding serum-free culture medium to the cells and incubating for an additional 10 min at 4°C. Cells were then washed twice with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5).

Surface biotinylated plasma membranes were isolated and resuspended in lysis buffer (3% CHAPS, 50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 7.5 containing 10 μ g/ml aprotinin A, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin), incubated for 2 h at 4°C with gentle agitation, and then cleared by centrifugation at 16,000 $\times$ g for 10 min at 4°C. Biotinylated proteins were

purified using a commercially available kit (ImmunoPure Immobilized Monomeric Avidin; Pierce).

Purification of P-selectin ligands

A P-selectin affinity column was prepared by incubating 2 mg of P-selectin-IgG-Fc fusion protein with 1 ml of protein-A agarose (Pierce) overnight at 4°C and then quenched with PBS buffer before washing with equilibration buffer (PBS containing 0.1% CHAPS, 1mM CaCl₂). A control column was prepared by coupling 2 mg of human IgG-Fc using same procedure. Biotinylated membrane proteins were diluted to a concentration of 1 mg/ml using equilibration buffer and pre-cleared with Protein-A agarose for 1 h at 4°C. Biotinylated membrane proteins (3 ml) were loaded onto either the P-selectin affinity column or the control IgG-Fc column and incubated on the column with gentle mixing for 2 h at 4°C. Unbound protein was eluted with equilibration buffer until absorbance at 280 nm reached baseline levels. Bound proteins were eluted with PBS containing 10 mM EDTA, 0.1% CHAPS, and 0.02% sodium azide. Eluted proteins were concentrated using Centrplus YM-3 centrifugal filter devices (Millipore, Bedford, MA).

Identification of polypeptides by in-gel cleavage and MS

Gel sections containing the major polypeptides eluted from the P-selectin affinity column, as judged by Coomassie-blue-staining, were excised and digested with 0.25 µg trypsin (Promega) for 8 h using a Progest In-gel Digester (Genomic Solutions, Ann Arbor, MI). Digested samples were lyophilized to dryness and resuspended in 50:50 (v/v) 0.2% formic acid:acetonitrile. Samples were then spotted onto a sample stainless steel MALDI plate and mixed with 0.3 µl of 33% saturated α -cyano-hydroxycinnamic acid. Mass spectrometric (MS) analyses, were then performed on an Applied Biosystems 4700 Proteomics Analyzer in the positive ion and reflector modes. The MS was internally calibrated using autolytic tryptic peptides and the MS/MS calibrated externally using the fragment ions of the angiotensin I M+H ion (m/z 1296.68). A focus mass of m/z 2000 was used for the MS acquisition. For the MS/MS analyses, 1000 V was used for the collision energy and argon used as the collision gas with a recharge threshold set at 1.0×10^{-7} torr. Protein identification was then performed by interrogating both the MS and the MS/MS using MASCOT and the entire NCBI non-redundant database. Search parameters included an allowance of two missed tryptic cleavages, a 0.06 Da mass tolerance for the MS data, a 0.1 Da mass tolerance for the MS/MS data, and an allowance for variable oxidation of methionine residues. The identity of the two Coomassie blue stained polypeptides with human nucleolin was assigned by comparison of the observed mass fingerprint with the predicted mass fingerprint of nucleolin in the database. In addition, relevant mass peaks were subjected to electrospray ionization for MS sequencing using an electrospray ionization tandem MS (QT, Micromass, U.K.) to confirm mass fingerprint analysis.

Adhesion Assay

Adhesion assays were carried out essentially as described [27]. Briefly, 96-well tissue culture clusters (Costar, Corning, NY) were coated by overnight incubation at 4°C with 20 µg/ml P-selectin-IgG-Fc in PBS or 10 µg/ml protein G (Pierce) in 50 mM Na₂CO₃/NaHCO₃, pH 9.5. Plates were washed with PBS and then incubated for 2 h at room temperature with 20 mg/ml heat-denatured BSA (Calbiochem, San Diego, CA) in presence or absence of 20 µg/ml a mAb anti-nucleolin or non-immune mouse IgG. After washing, 5×10^4 cells were added to each well for the times indicated in the figure legends. Attached cells were gently washed once with PBS, fixed, stained with 0.05% (w/v) crystal violet (Sigma) and destained. Dye solubilized with 10% acetic acid was quantified at 575 nm using a Spectra MAX 250 ELISA reader (Molecular Devices, Sunnyvale, CA).

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described [27] with non-permeabilized cells fixed with 3% paraformaldehyde. Immunofluorescence was documented with a LSM 410 inverted confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Omnichrome argon-krypton laser. Images were obtained with a Zeiss Plan-Apo 100X oil immersion objective (1.4 na).

Immunoblotting

Whole cell lysates or immunoprecipitates were resolved by 4-12% SDS-Tris polyacrylamide gel electrophoresis and then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) in Tris-glycine buffer containing 20% methanol. Proteins were detected by immunoblotting as described [27]. In some cases, PVDF membranes were stripped of bound antibodies using 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS for 30 min 60°C and then reprobed as described in figure legends.

RNA interference

The siRNA sequence chosen to target nucleolin was 5'-GGCAAAGCAUUGGUAGCAATT-3' (S1) in non-conserved regions of the nucleolin open reading frame (GenBank accession no. NM_005381). BLAST analysis showed no homology of the siRNA sequences to any other sequence in the Human Genome Database. The siRNA nucleotides were chemically synthesized and annealed by Ambion Inc. (Austin, TX). Nucleolin siRNAs (30 nM) were transfected in Colo-320 cells using siPORT NeoFX (Ambion). Scrambled siRNA for use as negative control was obtained from Ambion.

Results

PSGL-1 and CD24 do not function as P-selectin receptors in Colo-320 cells

We have reported that soluble P-selectin binding to Colo-320 cells increases cell adhesion to fibronectin through activation of the $\alpha 5 \beta 1$ integrin [27]. Consequently, we sought to identify and characterize the P-selectin receptors on these cells. We initially examined whether PSGL-1 and CD24, which have been most commonly identified as P-selectin counter-receptors [13, 30-34], were expressed by Colo-320 cells. As shown in Fig. 1A, both CD24 and PSGL-1 are expressed by Colo-320 cells, as judged by immunoblotting of whole cell lysates (lane 1). In control experiments, PSGL-1 was shown by confocal immunofluorescence microscopy to be expressed by Colo-320 cells exclusively intracellularly (Supplementary data, Fig 1). However, flow cytometry results showed that only CD24 (Fig. 1B), but not PSGL-1 (Fig. 1C), is expressed on the Colo-320 cell surface.

In order to identify whether CD24 could function as a P-selectin receptor in Colo-320 cells, cell surface proteins of intact cells were biotinylated using a membrane-impermeable sulfo-NHS-biotin, and the membrane fraction was isolated. Biotinylated cell membrane proteins were extracted and enriched by binding to, and eluting from, a P-selectin affinity column, and analyzed by immunoblotting with anti-CD24 or anti-PSGL-1 antibodies. Neither CD24 nor PSGL-1 could be detected in eluted proteins from the P-selectin affinity column (Fig 1A, lane 2). Furthermore, we used siRNA technology to assess the effect of knocking down expression of CD24 on P-selectin binding. Although we achieved up to 53% knockdown of CD24 expression, binding of P-selectin to Colo-320 cells was not affected (Supplementary data, Fig 2) apparently ruling out the possibility that CD24 may be functioning as a P-selectin receptor on these cells. Taken together, these results suggest that neither CD24 nor PSGL-1 has the properties expected of a cell surface P-selectin receptor on Colo-320 cells.

Cell-surface nucleolin is the most abundant protein eluted from P-selectin-affinity column

To identify the Colo-320 cell-surface P-selectin receptor(s), biotinylated proteins eluted from the P-selectin-affinity column were analyzed by avidin-HRP blotting and mass spectrometry (MS). Avidin-HRP blotting indicated that three cell-surface polypeptides with apparent relative molecular masses of 110, 90, and 45 kDa were eluted after binding to P-selectin-affinity column (Fig. 2A). No biotinylated proteins were detected from control IgG-Fc affinity column elutes. The 110-kDa polypeptide band was the most abundant, so it was chosen for further analysis by mass spectrometry (MS). After SDS/polyacrylamide gel electrophoresis and tryptic digestion, the MS fingerprint observed matched that for human nucleolin (Table 1). To confirm the identity of the 110-kDa polypeptide band as nucleolin (or a closely related protein), nucleolin was immunoprecipitated from the P-selectin-affinity elutes and analyzed by immunoblotting using the anti-nucleolin mAb, clone 4E2 (StressGen) and avidin-HRP blotting. As shown in Fig. 2B, both the 110 and 90-kDa polypeptide bands were immunoprecipitated as nucleolin from P-selectin-affinity column (upper panel), which corresponded to the two largest polypeptide bands observed from avidin-HRP blotting (Fig. 2A). However, avidin-HRP blotting of nucleolin immunoprecipitates detected only the 110-kDa polypeptide band (Fig. 2B, upper panel), which we have identified as nucleolin by MS. No bands were detectable in blotting analysis of nucleolin immunoprecipitates from IgG Fc-affinity column elutes. These results show that nucleolin was labeled on intact cells with membrane-impermeable biotin reagent, suggesting that a fraction of cellular nucleolin (or a nucleolin-like protein) is exposed on the Colo-320 cell surface.

To confirm that the detection of nucleolin expression on Colo-320 cells is not an artifact of the biotin reagent inadvertently crossing the plasma membrane, we examined the ability of Colo-320 cells to attach to anti-nucleolin mAbs (D3 and 4E2) immobilized on protein G-coated substrate. As shown in Fig. 2C, Colo-320 cells could attach to two different anti-nucleolin mAbs (D3 and 4E2) two-fold more than control mouse IgG. Furthermore, nucleolin expression could be detected by flow cytometry analysis on the surface of non-permeabilized Colo-320 cells with the same two anti-nucleolin mAbs, D3 and 4E2 (data not shown). All these results strongly suggest that nucleolin is expressed on the cell surface of Colo-320 cells.

Nucleolin is a P-selectin Receptor in Colo-320 cells

In order to show that nucleolin functions as a P-selectin receptor in intact living cells, and that the nucleolin/P-selectin association is not an artifact of cell lysis, we assessed the effect of anti-nucleolin mAbs on Colo-320 cell adhesion to immobilized P-selectin. As shown in Fig. 3A, Colo-320 cells could attach to immobilized P-selectin, and pretreatment of these cells with anti-nucleolin mAb (D3) could significantly ($P < 0.0019$) inhibit this attachment. In contrast, none of the anti-CD24 mAbs tested affects Colo-320 cell adhesion to P-selectin (data not shown). To confirm that cell-surface nucleolin functions as P-selectin receptor, we examined whether cross-linking of P-selectin-IgG-Fc bound to living, non-permeabilized Colo-320 cells in suspension induced co-clustering of cell-surface nucleolin. As judged by confocal immunofluorescence microscopy of cells, nucleolin and P-selectin were both co-localized on the surface of non-permeabilized Colo-320 cells. Subsequent cross-linking of P-selectin using anti-human-Fc antibodies resulted in co-clustering of nucleolin with the bound P-selectin (Fig. 3B). As a control, non-permeabilized cells were stained with DAPI. Nucleolin staining was not detected in the nucleus (Fig 3B), indicating that nuclear nucleolin is not accessible to P-selectin in non-permeabilized Colo-320 cells. These results strongly suggest that nucleolin is expressed on the surface of Colo-320 cells and is responsible for a significant amount of the interaction of Colo-320 cells with P-selectin.

P-selectin binding induces tyrosine phosphorylation of cell-surface nucleolin

We have previously shown that incubation of P-selectin with Colo-320 cells leads to changes in the phosphorylation state of intracellular proteins [27]. To determine whether nucleolin can play a role in P-selectin-initiated signal transduction, we first examined whether nucleolin could be phosphorylated on tyrosine residues in a P-selectin dependent manner. As shown in Fig. 4A, only nucleolin immunoprecipitated from Colo-320 cells that had been incubated with P-selectin-IgG-Fc showed a marked increase of tyrosine phosphorylation, as judged by immunoblotting with an anti-phosphotyrosine antibody. In contrast, nucleolin immunoprecipitated from untreated control cells or cells treated with human-IgG was not tyrosine phosphorylated. This tyrosine phosphorylation of nucleolin was specifically caused by P-selectin treatment of the Colo-320 cells. No tyrosine phosphorylation of nucleolin could be observed from cells incubated with either E-selectin-IgG-Fc or L-selectin-IgG-Fc, as judged by immunoblotting with anti-phosphotyrosine mAb (Fig 4A, compare lanes 4 and 5 with lane 3).

Nucleolin can be expressed in the nucleus, cytoplasm, and plasma membrane [35,36]. In order to determine whether P-selectin could specifically mediate phosphorylation of cell-surface nucleolin, cells were incubated with P-selectin or left untreated and fractionated. Nucleolin immunoprecipitated from each cellular fraction was analyzed by immunoblotting with anti-phosphotyrosine mAb. As shown in Fig. 4B, nucleolin was found to be expressed in the nuclear and membrane fractions of Colo-320 cells. Subsequent incubation of these cells with P-selectin resulted in increased tyrosine phosphorylation specifically of nucleolin expressed in the membrane fraction. In contrast, there was no detectable increase of tyrosine phosphorylation of nuclear nucleolin. Interestingly, we also observed an increase of expression of nucleolin in the cytoplasmic compartment resulting from P-selectin binding. As a control, lysates from each cellular fraction were analyzed by immunoblotting with antibodies for histone 3 and Na⁺/K⁺-ATPase α 1 corresponding to the nuclear and plasma membrane markers respectively. Na⁺/K⁺-ATPase and histone 3 were only detected in their corresponding cellular compartment, showing that there was no detectable cross contamination between the membrane and nuclear fractions (Fig 4C). Thus, our results suggest strongly that plasma-membrane nucleolin can function as a signal transducing receptor for P-selectin in Colo-320 cells and this signal transduction occurs via tyrosine phosphorylation.

Nucleolin is present in a P-selectin-mediated signaling complex

We have recently reported that P-selectin binding to Colo-320 cells induces the formation of a signaling complex containing both p38 MAPK and PI3-K [27]. In order to determine whether nucleolin could form part of this signaling complex, we analyzed whether incubation of Colo-320 cells with P-selectin could result in the co-immunoprecipitation of nucleolin, p38 MAPK, and PI 3-K. First, p38 MAPK and nucleolin were immunoprecipitated from cell lysates derived from untreated cells and P-selectin stimulated cells, and immunoprecipitates were analyzed by immunoblotting. As shown in Fig 5A, nucleolin was co-immunoprecipitated with anti-p38 MAPK antibodies from lysates derived from P-selectin-incubated cells, but not from lysates from control cells not incubated with P-selectin (Fig. 5A, lanes 1 and 2). Similarly, p38 MAPK was co-immunoprecipitated from cell lysates with anti-nucleolin antibody from P-selectin incubated cells, but not from untreated cells (Fig. 5A, lanes 3 and 4). We also examined whether nucleolin and PI3-K could be co-immunoprecipitated from cells lysates after P-selectin incubation. As shown in Fig. 5B, when the p110 subunit of PI 3-K was immunoprecipitated from lysates prepared from untreated cells and P-selectin treated cells, and the resulting precipitates were immunoblotted with anti-nucleolin antibody, nucleolin was only detected in the PI 3-K immunoprecipitates from P-selectin-treated cells, but not from untreated cells (Fig. 5B, lanes 1 and 2). Likewise, PI 3-K p85 subunit was co-immunoprecipitated with anti-nucleolin antibody from P-selectin treated, but not from

untreated cells (Fig. 5B, lanes 3 and 4). These results suggest that nucleolin, p38 MAPK, and PI 3-K can form a P-selectin-mediated signaling complex.

In order to determine if tyrosine phosphorylation of nucleolin is required for the formation of the P-selectin-mediated nucleolin/PI 3-K/p38 MAPK signaling complex, we analyzed the effect of the general tyrosine kinase inhibitor, genistein, in co-immunoprecipitation experiments. Genistein blocked both the P-selectin-mediated nucleolin tyrosine phosphorylation (Fig. 5C, lanes 1-3) and the nucleolin/PI 3-K co-immunoprecipitation (Fig. 5C, lanes 4-6), but not the nucleolin/p38 MAPK co-immunoprecipitation (Fig. 5C, lanes 5 and 6). These results suggest that tyrosine phosphorylation of nucleolin is required for the interaction of nucleolin with PI 3-K, but not for the interaction of nucleolin and p38 MAPK after P-selectin binding to Colo-320 cells.

Nucleolin regulates the P-selectin-mediated PI 3-K/p38 MAPK protein complex formation

We next determined if nucleolin is necessary for the formation of the signaling complex containing PI3-K/ p38 MAPK signaling complex by using an siRNA approach to knockdown the expression of nucleolin. Immunoblot analyses of total cell lysates using anti-nucleolin antibody showed that expression of total nucleolin could be reduced by at least 70% in cells transfected with nucleolin siRNAs compared with control cells (Fig. 6A). As judged by flow cytometry analysis, P-selectin binding to Colo-320 cells was significantly decreased ($P<0.007$) in cells transfected with nucleolin siRNAs compared with that of control cells (Fig. 6B). These results also confirmed that nucleolin is a P-selectin receptor on Colo-320 cells.

To determine the role of nucleolin in the formation of P-selectin-mediated PI3-K/p38 MAPK protein complex, p38 MAPK was immunoprecipitated from lysates of transfected cells with nucleolin siRNAs or control cells that were only in presence transfection reagent (Mock) and analyzed by immunoblotting with anti-PI 3-K and anti-nucleolin antibodies. As shown in Fig. 6C, PI 3-K and nucleolin were present in p38 MAPK immunoprecipitates from P-selectin-stimulated cells that were mock-transfected (Mock). However, P-selectin-mediated PI 3-K and nucleolin co-immunoprecipitation with p38 MAPK was completely blocked in the cells transfected with nucleolin siRNAs (Fig. 6C). These results suggest that nucleolin is required for recruiting of both PI3K and p38 MAPK into a P-selectin-mediated signaling complex.

Discussion

We have identified tumor cell-surface nucleolin (or a nucleolin-like protein) as a P-selectin receptor that can also function in signal transduction by mediating the formation of a signaling complex. We have found that cell-surface nucleolin can become tyrosine phosphorylated as a result of P-selectin binding, and this modification is required for the formation of a signaling complex that includes nucleolin, PI 3-K, and p38 MAPK. Previously, we have shown that P-selectin binding to Colo-320 cells can specifically activate the $\alpha 5 \beta 1$ integrin, which results in an increase of cell adhesion on fibronectin substrates [27]. We suggest that cell-surface nucleolin can function as both a signaling receptor and an adaptor protein in the signal transduction pathway important for P-selectin-mediated integrin activation.

In the present study, three cell surface polypeptides of molecular masses of 110, 90, and 45 kDa were detected as possible P-selectin binding proteins by avidin-HRP blotting analysis of proteins purified from biotinylated Colo-320 cell plasma membranes using P-selectin-affinity chromatography. None of these polypeptides was recognized by the antibodies for PSGL-1 and CD24, molecules well characterized as P-selectin receptors. Furthermore, specific knockdown of expression of CD24 had no detectable effect on P-selectin binding to Colo-320 cells. We also find that PSGL-1 is expressed intracellularly but is not expressed on the surface of Colo-320 cells. These results suggest neither CD24 nor PSGL-1 has the characteristics

required of a P-selectin receptor for these cells. We focused our study on the 110-kDa polypeptide because it was the most abundant protein eluted from a P-selectin affinity chromatography, suggesting that this polypeptide could be the major receptor for P-selectin on Colo-320 cells. Moreover, other studies have also reported a 110-kDa polypeptide as a P-selectin binding protein purified from human malignant melanoma cell line NKI-4 [7] and human small cell lung carcinoma cell line NCI-H345 [37] using P-selectin affinity chromatography. We identified the 110-kDa polypeptide as the protein, nucleolin, by MS and immunoassays. The 90-kDa polypeptide was also identified as nucleolin, but it was poorly biotinylated compared to the 110-kDa polypeptide, suggesting that either the 90-kDa polypeptide is not expressed at high levels on cell surface or it may be a proteolytic product of nucleolin. Different forms of nucleolin have been described as a result of specific proteolytic cleavage [38].

Nucleolin has been previously characterized as a protein highly expressed in the nucleus [35]. Therefore, we confirmed and validated our finding that nucleolin is also expressed on the surface of Colo-320 cells and its function as a receptor for P-selectin by five different criteria: (1) The charged reagent sulphosuccinimidobiotin, which is unable to penetrate the plasma membrane, could modify nucleolin on intact, living cells; (2) Colo-320 cells could attach to anti-nucleolin mAbs immobilized on a solid substrate; (3) An anti-nucleolin antibody significantly inhibited Colo-320 cell adhesion to immobilized P-selectin; (4) Cross-linking of P-selectin bound to Colo-320 cells induced co-clustering of cell-surface nucleolin; and (5) decreasing nucleolin expression using siRNA produced a significant reduction in P-selectin binding to Colo-320 cells. Taken together all these results strongly suggest that nucleolin is expressed on cell-surface and functions as a receptor for P-selectin in Colo-320 cells. Furthermore, these five independent approaches make it highly unlikely that detection of nucleolin is an artifact of internalization of NHS-sulfo-biotin or soluble antibodies, or of cell lysis. Additionally, the expression of nucleolin on the cell surface is supported by several previously published studies. For example, nucleolin has been localized on the cell surface of aggressive tumors in vivo [35,39-47] and been found to function as a receptor for a number of ligands including apo B- and apo E-containing lipoprotein, laminin-1, fructosyllysine, factor J, midkine, L-selectin, HIV, and coxsackie B viruses [35,39-47]. Here, we have also shown that cell-surface nucleolin is a P-selectin signaling receptor, an apparently novel finding. Cell-surface nucleolin can be specifically tyrosine phosphorylated as a result of P-selectin binding to Colo-320 cells. In contrast, we are unable to detect tyrosine phosphorylation of nucleolin expressed in the cytoplasm or the nucleus. We also found that tyrosine phosphorylated cell-surface nucleolin could participate in the formation of a P-selectin-induced complex with p38 MAPK/PI 3-K, as judged by co-immunoprecipitation experiments. Moreover, knock down of expression of nucleolin using siRNA blocked the P-selectin-mediated PI 3-K/p38 MAPK complex formation showing that nucleolin is a critical component in this signaling complex. Previously, we have demonstrated that P-selectin stimulation induce the $\alpha 5 \beta 1$ integrin activation through the PI 3-K and p38 MAPK signaling pathways –linked by a p38 MAPK/PI 3-K signaling complex [27]. Thus, our results are consistent with the possibility that cell-surface nucleolin plays a direct role in P-selectin-mediated integrin activation signaling pathways.

We found that tyrosine kinase activity was important for the P-selectin-mediated nucleolin/PI 3-K interaction, suggesting that tyrosine-phosphorylated nucleolin might participate in PI3-K activation. A possible mechanism of this activation could involve an interaction of a phosphotyrosine containing protein with the SH2 domain of p85 inducing a conformational change in this regulatory subunit that activates the p110 catalytic subunit [48]. This mechanism is supported by analysis of amino acid sequences of nucleolin to predict potential tyrosine phosphorylation sites (www.cbs.dtu.dk/services/NetPhos/) [49] and functional sites in eukaryotic proteins (www.elm.eu.org) [50]. These analyses suggest that nucleolin may contain

four potential phosphorylation sites (Y351, Y402, Y495 and Y525) and three motifs that may function as ligands for Src homology 2 (SH2) domains (positions 351-354, 433-436, and 463-466). In fact, analysis of the amino acid sequence of nucleolin yields an overlap between in the possible tyrosine phosphorylation site Y351 and the SH2 ligand domain in the position 351-354. This finding suggests that nucleolin may contain at least a functional SH2 ligand domain which possible is recognized by SH2 domain of PI 3-K p85 to induce PI 3-K binding and activation. The nucleolin/PI-3K interaction has been reported to be induced in other two different cellular systems described in the literature. In normal B lymphocytes, CD21 activation induced tyrosine phosphorylation of nucleolin which interacted with Src homology 2 (SH2) domain of p85 subunit of PI3-K [51]. In endothelial cells, the application of fluid stress also induced a nucleolin/PI-3K interaction [52] although the mechanism of direct tyrosine phosphorylation of nucleolin by fluid stress was not determined. Therefore, the nucleolin/PI 3-K interaction is apparently not limited to P-selectin-mediated signaling pathways, suggesting that nucleolin could be a generally important in PI-3K signaling pathway.

Analysis of the amino acid sequence of nucleolin also suggests that it does not contain intrinsic tyrosine kinase capacity, suggesting that its phosphorylation is mediated by an, as yet, unidentified tyrosine kinase. It has been previously reported that CD21 plays roles in both nucleolin phosphorylation through of pp60Src activation and linkage to PI 3-K activation in normal B lymphocytes [53]. However, we were unable to detect pp60Src activation or nucleolin/pp60Src co-immunoprecipitation in P-selectin-stimulated Colo-320 cells (Reyes and Akiyama, unpublished data). Since the form of nucleolin that is specifically phosphorylated is associated with the plasma membrane and appears to function as a P-selectin receptor, we hypothesize that a cell-surface kinase may be involved.

P-selectin has been found to play important roles in the hematogenous phase of metastasis by: (1) promoting the initial attachment of cancer cells to the blood vessel endothelium [13-15, 31], (2) mediating the interactions between tumor cells and platelets to form emboli to facilitate the arrest of cancer cells [16,17,54,55], (3) regulating signals that effect cell proliferation of cancer cells [20], and (4) regulating the integrin-mediated colon cell adhesion [27]. We have no data to suggest that these distinct roles of P-selectin in the metastasis are mediated by nucleolin and, in fact, we hypothesize that different P-selectin receptors may mediate different steps in metastasis. Previously published studies suggest that different human tumor cells could have different P-selectin receptors [7,31,37,56,57]. Thus it is likely that each different P-selectin receptors could each mediate a specific P-selectin function in the process of metastasis. Our results are consistent with cell-surface nucleolin playing an important role in activation of integrin-mediated carcinoma cell adhesion resulting from interactions with P-selectin.

Our results are also consistent with the increasing evidence suggesting that cell-surface nucleolin may play an important role in the regulation of tumor cell biology in response to environmental cues. Previously published studies have suggested that cell surface nucleolin can function as a marker for angiogenesis and play a key role in the angiogenic process [39, 58]. Nucleolin has also been reported to be expressed on the endothelial cell surface only during the process of tumor angiogenesis; specifically, cell-surface nucleolin expression in endothelial cells appeared to be required for endothelial cell motility and formation of the tubular structures observed in the angiogenic process.

Our results suggest that cell-surface nucleolin also regulates integrin-mediated tumor cell adhesion. This conclusion is supported by a recently published study showing that cell-surface nucleolin functions as the hepatocyte growth factor (HGF) signaling receptor that regulates the adhesion of PC3 and LNCaP prostate cancer cells to laminin [59]. Taken together, these results provide clear evidence that cell-surface nucleolin is involved in the modulation of tumor cell

behavior and suggests that nucleolin may be an attractive potential target for therapeutics that inhibit the progress of metastatic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. P-Selectin Glycoprotein Ligand 1 and CD24 are expressed by Colo-320 cells, but do not function as P-selectin receptors

(A) Total Colo-320 cell lysates (lane 1) and P-selectin ligands purified by affinity chromatography from Colo-320 cells (lane 2) were resolved by 4-12% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with either anti-P-selectin glycoprotein ligand 1 (PSGL-1) or anti-CD24 antibodies. (B and C) Colo-320 cells were incubated with either 10 μ g/ml anti-CD24 antibody (Panel B, blue line), anti-PSGL-1 (Panel C, blue line), or 10 μ g/ml non-immune mouse IgG (gray histograms), followed by FITC-conjugated anti-mouse IgG-Fc F(ab)₂, and analyzed by flow cytometry.

Figure 2. Identification of nucleolin (or a nucleolin-like protein) as the major protein eluted from P-selectin affinity and as a cell surface protein in Colo-320 cells

Cell-surface proteins from intact Colo-320 cells were labeled covalently with membrane impermeable sulphosuccinimidobiotin. Biotinylated plasma membrane proteins were purified and then incubated with, and eluted from, either a P-selectin affinity column or a control IgG-Fc column. (A) Proteins eluted from P-selectin IgG-Fc affinity column (Psel) or IgG-Fc affinity column (Fc) were detected by blotting with HRP-conjugated streptavidin. (B) Nucleolin was immunoprecipitated from the proteins eluted from P-selectin-IgG-Fc affinity column (Psel) or IgG-Fc affinity column (Fc) and analyzed by blotting with either anti-nucleolin antibody (upper panel) or HRP-conjugated streptavidin (lower panel). The arrowhead indicates the band

corresponding to nucleolin. (C) Protein G-coated microwells were incubated with 2% denatured BSA/PBS in absence or presence of 20 µg/ml anti-nucleolin antibodies (D3 or 4E2) or non-immune mouse IgG. Colo-320 cells (5×10^4 cells) in 2% denatured BSA/PBS were allowed to attach to antibodies immobilized for 30 min at 4°C. Error bars indicate standard deviation.

Figure 3. Cell-surface nucleolin functions as P-selectin binding protein in Colo-320 cells
 (A) Colo-320 cells were allowed to attach to immobilized BSA (white bars) or P-selectin (black bars) substrates for 60 min in the absence (-) or presence of 20 μ g/ml anti-nucleolin mAb (D3) or non-immune mouse IgG for 60 min at 37°C. Error bars indicate standard deviation. (B) Colo-320 cells were incubated with P-selectin IgG-Fc chimera at 4°C for 45 min. After washing, cell were plated on polylysine-coated cover slips in the presence or absence of anti-human IgG-Fc (cross-linker) and incubated for 10 min at 37°C, washed, and fixed. The localization of nucleolin on non-permeabilized cells was determined by staining with antibody against nucleolin and with Alexa 488 goat anti-mouse IgG-Fc (green). P-selectin was localized

with Alexa 565 goat anti-human IgG-Fc (red). The nucleus was stained with DAPI (blue). Fluorescent images were combined to determine colocalization (yellow). Bar = 10 μ M.

Figure 4. P-Selectin induces an increase of tyrosine phosphorylation of cell surface nucleolin
 (A) Colo-320 cells were incubated in suspension without (lane 1) or with 20 µg/ml human-IgG (Fc) (lane 2) or P-, L-, or E-selectin-IgG-Fc (lanes 3, 4, and 5, respectively) for 5 min at 37°C. Cells were then lysed and nucleolin was immunoprecipitated and analyzed by immunoblotting with anti-phosphotyrosine antibody (upper panel). After stripping bound antibodies, the membrane was probed for total nucleolin (lower panel). The arrowheads indicate the positions of the nucleolin bands. (B) Cells incubated with or without 20 µg/ml P-selectin-IgG-Fc (Psel) for 5 min at 37°C were fractionated into membrane, cytoplasmic, and nuclear fractions. Nucleolin was immunoprecipitated from each cellular fraction and analyzed by immunoblotting with anti-phosphotyrosine antibody (upper panel). After stripping bound

antibodies, the membrane was probed for total nucleolin (lower panel). (C) Total lysates from each cell fraction from panel B were analyzed by immunoblotting with antibodies for a plasma membrane marker (anti- Na^+/K^+ -ATPase $\alpha 1$) and a nuclear marker (anti-histone 3) to show absence of cross-contamination of the membrane and nuclear fractions.

Figure 5. Nucleolin co-immunoprecipitates with p38 MAPK and PI 3-K on a P-selectin-dependent manner

Colo-320 cells were incubated in suspension without (-) or with 20 μ g/ml P-selectin-IgG-Fc (Psel) for 5 min. Then, p38 MAPK, nucleolin, or PI 3-K p110 were immunoprecipitated. (A) p38 MAPK and nucleolin immunoprecipitates were analyzed by immunoblotting with anti-nucleolin, anti-phospho-p38 MAPK, and anti-total p38 MAPK. (B) PI 3-K and nucleolin immunoprecipitates were analyzed by immunoblotting with anti-nucleolin and anti-PI 3-K (p85). (C) Cells were pretreated with or without 200 μ M genistein for 30 min at 37°C prior to P-selectin stimulation. Nucleolin immunoprecipitates from treated and untreated cell lysates were analyzed by immunoblotting for phosphotyrosine, p38 MAPK, or PI3-K. After stripping

bound antibodies, the same membranes were reprobed for total nucleolin. The arrowhead indicates the nucleolin band.

Figure 6. Nucleolin expression is required for formation of the P-selectin-mediated PI 3-K/p38 MAPK signal complex formation

Colo-320 cells were transfected with nucleolin siRNAs (NCL), control siRNAs (C), or incubated under the same conditions in the presence (M) or absence (B) of transfection reagent. After culturing the cells for 48 h after transfection, (A) cells were lysed and analyzed by immunoblotting with anti-nucleolin antibody. After stripping bound antibodies, the same membranes were reprobed for GAPDH (lower panels). (B) Transfected Colo-320 cells were incubated with 20 μ g/ml P-selectin-IgG-Fc or with 20 μ g/ml human IgG-Fc, followed by FITC-conjugated anti-human IgG-Fc F(ab)₂, and binding was assessed by flow cytometry. The % P-Selectin binding was calculated using the following formula: % P-selectin binding = Mean

fluorescence ($\text{Psel}_X\text{-Fc}_X$) / ($\text{Psel}_{\text{Buffer}}\text{-Fc}_{\text{Buffer}}$) * 100, X= Transfected cells with nucleolin siRNAs, control siRNAs, or only incubated with presence (Mock) or absence (Buffer). (C) p38 MAPK was immunoprecipitated from transfected Colo-320 cells stimulated with P-selectin (Ps) or control, unstimulated (-), cells. p38 MAPK immunoprecipitates were analyzed by immunoblotting with anti-PI 3-K or anti-nucleolin. The membranes were probed for total p38 MAPK (lower panels) after stripping bound antibodies.

Table 1
MS analysis of the 110-kDa protein

The 110-kDa band purified on a P-selectin affinity column was analyzed by MS after in gel trypsin digestion and desalting. Peptides identified by peptide mass-fingerprint analysis are listed. Protein identification was then performed by interrogating both the MS and the MS/MS using MASCOT and the entire NCBI non-redundant database (matrix-assisted laser-database accession number P19338).

Nucleolin Residues	Peptide	Observed Mass	Predicted Mass
298-318	VEGTEPTTAFNLFVGNLNFNK	2311.153	2311.147
325-333	TGISDVFAK	936.460	936.491
334-342	NDLAVVDVR	999.531	999.535
348-362	KFGYVDFESAEDLEK	1775.841	1775.825
349-362	FGYVDFESAEDLEK	1647.752	1647.730
371-377	VFGNEIK	805.412	805.433
404-420	VTQDELKEVFEDAAEIR	1991.009	1990.984
411-420	EVFEDAAEIR	1177.572	1177.561
430-437	GLAYIEFK	939.486	939.506
458-467	SISLYYTGEK	1159.557	1159.575
468-474	GQNQDYR	879.386	879.383
487-508	TLVLSNLSYSATEETLQEVFEK	2500.270	2500.257
509-521	ATFIKVPQNQNGK	1443.759	1443.783
514-521	VPQNQNGK	883.467	883.451
524-537	GYAFIEFASFEDAK	1593.720	1593.734
555-561	LELQGPR	811.453	811.455
578-597	GLSED'TTEETLKESFDGSR	2199.051	2199.017
590-597	ESFDGSR	895.417	895.403
600-610	IVTDRETGSSK	1191.622	1191.609
611-624	GFGFVDFNSEEDAK	1560.680	1560.673