Cellular Signalling 35 (2017) 1–15

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

As human lung microvascular endothelia achieve confluence, src family kinases are activated, and tyrosine-phosphorylated p120 catenin physically couples NEU1 sialidase to CD31

Sang W. Hyun[a,b,1], Anguo Liu[a,b,1], Zhenguo Liu[a,b], Erik P. Lillehoj[c], Joseph A. Madri[d], Albert B. Reynolds[e], Simeon E. Goldblum[a,b,*]

[a] U.S. Department of Veterans Affairs, Baltimore, MD 21201, United States
[b] Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, United States
[c] Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD 21201, United States
[d] Department of Pathology, Yale University School of Medicine, New Haven, CT 06520, United States
[e] Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN 37232, United States

ARTICLE INFO

Keywords:
Endothelial cell
Lung
Neuraminidase
Platelet endothelial cell adhesion molecule (PECAM-1)
Sialic acid

ABSTRACT

In postconfluent human pulmonary microvascular endothelial cell (HPMEC)s, NEU1 sialidase associates with and desialylates the src family kinase (SFK) substrate, CD31, and disrupts angiogenesis. We asked whether the NEU1-CD31 interaction might be SFK-driven. We found that normalized phospho-SFK (PY416) signal is increased in postconfluent HPMECs compared to subconfluent cells and prior SFK inhibition with PP2 or SU6656 completely blocked NEU1 association with and desialylation of CD31. Prior silencing of each of the four SFKs expressed in HPMECs, as well as CD31, dramatically reduced confluence-induced SFK activation. No increases in tyrosine phosphorylation of NEU1 or CD31 were detected. However, in postconfluent cells, we found increased tyrosine phosphorylation of a 120 kDa protein that was identified as p120 catenin (p120ctn). Prior silencing of c-src, fyn, or yes each reduced p120ctn phosphorylation. Prior knockdown of p120ctn prevented NEU1-CD31 association in both co-immunoprecipitation and pull-down assays. In these same assays, p120ctn associated with each of the four HPMEC-expressed SFKs as well as CD31 and NEU1. The CD31-p120ctn interaction was SFK-dependent whereas the NEU1-p120ctn interaction was not. Using purified recombinant binding partners in a cell-free system, direct protein-protein interactions between NEU1, CD31, and p120ctn were detected. Our combined data indicate that as HPMECs achieve confluence and CD31 ectodomains become homophilically engaged, multiple SFKs are activated to increase tyrosine phosphorylation of p120ctn, which in turn, functions as a cross-bridging adaptor molecule that physically couples NEU1 to CD31, permitting NEU1-mediated desialylation of CD31. These findings establish a SFK-driven, p120ctn-dependent mechanism for NEU1 recruitment to CD31.

1. Introduction

The microvascular endothelial surface that lines the intravascular space is highly sialylated [1,2]. Sialic acid (SA) residues are 9-carbon sugars carboxylated on the C1 position that occupy the outermost positions of oligosaccharide chains tethered to glycoproteins and glycolipids expressed on the cell surface [3]. The subterminal sugars to which SA is usually coupled are galactose and *N*-acetylgalactosamine. In most instances, the C2 position of SA is coupled to underlying galactose via α2,3- or α-2,6-linkage and/or to *N*-acetylgalactosamine via α-2,6-linkage. These terminally positioned, negatively charged SA residues are strategically positioned to influence intermolecular and

Abbreviations: aa, amino acid; Ad, adenovirus; CD, cytoplasmic domain; EC, endothelial cell; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; GST, glutathione S-transferase; HPMEC, human pulmonary microvascular endothelial cell; HRP, horseradish peroxidase; MOI, multiplicity of infection; MUC1, mucin-1; NEU, neuraminidase/sialidase; p120ctn, p120 catenin; PECAM-1, platelet endothelial cell adhesion molecule-1; PPCA, protective protein/cathepsin A; PNA, peanut agglutinin; PTP, protein tyrosine phosphatase; pTyr, phosphotyrosine; PVDF, polyvinylidene difluoride; SA, sialic acid; SFK, src family kinase; SH2, src homology 2; SHP, src homology domain 2-containing tyrosine phosphatase; ST, sialyltransferase; TBS-T, Tris-buffered saline-Tween 20; TLR, toll-like receptor; VE-cadherin, vascular endothelial-cadherin

* Corresponding author at: University of Maryland School of Medicine, 20 Penn Street, Room 351, Baltimore, MD 21201, United States.
E-mail address: sgoldblu@mbrc.umaryland.edu (S.E. Goldblum).

[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.cellsig.2017.03.014
Received 19 September 2016; Received in revised form 22 March 2017; Accepted 22 March 2017
Available online 24 March 2017
0898-6568/

intercellular interactions through their incorporation into specific recognition motifs [4] or masking cryptic binding sites via steric hindrance, electrostatic repulsion, and/or changes of the glycan chain conformation or flexibility [5,6]. Surface sialylation influences multiple endothelial cell (EC) functions, including their adhesiveness for circulating leukocytes [4,5,7] and the underlying extracellular matrix [2,8], EC migration into a wound [8,9], and EC capillary-like tube formation [8,10]. Sialylated molecules at the EC surface also regulate components of the clotting [11] and complement [12] pathways.

The sialylation state of a specific molecule is dynamically and coordinately regulated through the opposing catalytic activities of sialyltransferase (ST)s [13] and neuraminidase/sialidase (NEU)s [14,15]. An ST family of at least 15 members catalyzes transfer of SA residues to terminal positions on glycan chains [13]. These STs can be classified based on the specific SA linkage they synthesize and the sugar to which they transfer the SA. In contrast, NEUs counter-regulate sialylation through hydrolysis of the linkage between terminal SAs and their subterminal sugars [14,15]. Four human NEUs have been identified, NEU1, −2, −3, and −4 [14,15].

We now have established the predominant NEU expressed in vascular endothelia as NEU1 [9]. NEU1 is a ~45.5 kDa (415 amino acid [aa]) protein that resides in a multienzyme complex comprised of NEU1, protective protein/cathepsin A (PPCA), and β-galactosidase [14,15]. PPCA functions as an intracellular chaperone and transport protein that is required for proper folding, stability, oligomerization, and activation of NEU1 [16,17]. Although NEU1 was discovered as a lysosomal enzyme [16], it also can translocate to the cell surface where it associates with several multi-receptor signaling complexes, including Toll-like receptor (TLR)4/CD14/MD2 [18], the elastin receptor complex [19], and epidermal growth factor receptor (EGFR)/mucin-1 (MUC1) [20]. However, the mechanism(s) through which NEU1 is recruited to the cell surface is unclear. More recently, we found that in human pulmonary microvascular EC (HPMEC)s, CD31, also known as platelet EC adhesion molecule (PECAM)-1, contains α2,6-linked SA residues, and in the postconfluent state, when the ectodomains of CD31 are homophilically engaged, NEU1 is recruited to and desialylates CD31 [8]. NEU1 restrained EC migration and disrupted EC capillary-like tube formation through its ability to desialylate its substrate, CD31 [8]. In the current studies, we have asked what signaling events are triggered through EC-EC contact that might provoke NEU1 recruitment to CD31.

2. Materials and methods

2.1. HPMEC culture

HPMECs (Promocell, Heidelberg, Germany) were cultured in EC growth medium (MV-2, Promocell) containing EC growth medium supplement mix (Promocell), as described [8]. HPMECs were studied in passages 4–7. In most experiments, the HPMECs were cultured to subconfluent (< 50% confluent) and postconfluent (100% confluent) states.

2.2. Detection of src family kinase (SFK) activity by phospho-SFK (PY416) immunoblotting

Subconfluent and postconfluent HPMECs were lysed and the lysates processed for immunoblotting with rabbit polyclonal anti-human phospho-SFK (PY416) antibody (Cell Signaling Technology, Danvers, MA) followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Cell Signaling Technology), as described [21,22]. To confirm equivalent protein loading and transfer, blots were stripped with 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, and reprobed with murine monoclonal anti-β-tubulin antibody (Invitrogen, Carlsbad, CA) followed by HRP-conjugated horse anti-mouse IgG antibody (Cell Signaling Technology). Blots were developed by enhanced chemiluminescence (ECL). Densitometric quantification of phospho-SFK signal in each lane was normalized to β-tubulin signal in the same lane in the same stripped and reprobed blot.

2.3. Manipulation of SFK activity or SFK, NEU1, CD31, vascular endothelial (VE)-cadherin, and p120ctn expression in HPMECs

To pharmacologically block SFK catalytic activity, HPMECs were preincubated for 2 h with either of two SFK-selective inhibitors, SU6656 (10 μM) or PP2 (10 μM) (Calbiochem, San Diego, CA). To overexpress NEU1, HPMECs were transiently infected with an adenovirus encoding FLAG-tagged NEU1 (Ad-NEU1-FLAG) at a multiplicity of infection (MOI) of 100 or 200, as described [8]. After 48 h, the NEU1-overexpressing ECs were processed for FLAG (NEU1) immunoblotting and peanut agglutinin (PNA) lectin blotting. To silence NEU1, SFKs, CD31, p120ctn, or VE-cadherin expression, HPMECs were transfected with siRNA duplexes designed to specifically target NEU1, c-src, fyn, yes, lyn, CD31, p120ctn, or VE-cadherin, or irrelevant control siRNA duplexes not corresponding to any known sequence in the human genome (Dharmacon, Lafayette, CO), as described [8]. For transfection, 5.0×10^5 ECs were centrifuged (200 × g for 10 min), and the cell pellet was resuspended in 100 μl of Amaxa Nucleofactor solution (Amaxa Biosystems/Lonza, Walkersville, MD) with 2.7 μg of siRNA duplexes. The EC-siRNA mixture was transferred to an Amaxa-certified cuvette and subjected to programmed electroporation (program S-005; Amaxa Biosystems/Lonza). The transfected ECs were cultured for 48 h, after which they were lysed, and the lysates processed for immunoblotting with antibodies against the targeted protein of interest. For selected experiments, transfection was performed with the Lipofectamine 2000 reagent (Invitrogen). In other experiments, HPMECs were transiently infected with Ad-NEU1-FLAG at an MOI of 100. After 48 h, NEU1-targeting or control siRNAs were transfected into the HPMECs overexpressing FLAG-tagged NEU1. After 48 h, the transfected cells were lysed and the lysates processed for immunoblotting with murine monoclonal anti-FLAG (NEU1) antibody (Cell Signaling Technology). To control for protein loading and transfer, blots were stripped and reprobed for β-tubulin. Infected and transfected cells were studied for phospho-SFK immunoblotting, co-immunoprecipitation and in vitro pull-down assays.

2.4. CD31/NEU1-FLAG, CD31/p120ctn, NEU1-FLAG/p120ctn, and SFK/p120ctn co-immunoprecipitation assays

For the CD31/NEU1 co-immunoprecipitation assays, HPMECs pre-infected with Ad-NEU1-FLAG (MOI = 100) were cultured to subconfluent and postconfluent states, lysed, and the lysates immunoprecipitated with murine monoclonal anti-CD31 antibody (Cell Signaling Technology). The CD31 immunoprecipitates were processed for immunoblotting with anti-FLAG (NEU1) antibody, as described [8]. To control for protein loading and transfer, the blots were stripped and reprobed for CD31. Densitometric quantitation of FLAG (NEU1) signal was normalized to CD31 signal in the same lane on the same stripped and reprobed blot. For the CD31/p120ctn co-immunoprecipitation assays, lysates of subconfluent and postconfluent HPMECs were immunoprecipitated with murine monoclonal anti-p120ctn antibody (BD Biosciences, San Jose, CA) and the p120ctn immunoprecipitates processed for CD31 immunoblotting. Similarly, CD31 and FLAG (NEU1) immunoprecipitates were processed for p120ctn immunoblotting. In selected experiments, the SFK immunoprecipitates were processed for p120ctn immunoblotting. In each case, immunoprecipitates were probed for putative binding partners, followed by stripping and reprobing with the immunoprecipitating antibody for normalization.

2.5. PNA lectin blotting of CD31

HPMECs and HPMECs pretreated with SU6656 or PP2, were cultured to subconfluence or postconfluence and lysed, and the lysates