Identification of human endomucin-1 and -2 as membrane-bound *O*-sialoglycoproteins with anti-adhesive activity[1]

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Abstract Using a signal sequence trap method and database search, we identified a series of human cDNAs encoding two structurally related type I membrane proteins of ~25 kDa with multiple glycosylation motifs. These genes, termed *endomucin-1/-2*, are expressed in several human tissues including heart, kidney, and lung. Exogenously expressed human endomucin-1/-2 proteins were modified into 80–120 kDa glycoproteins, which were susceptible to *O*-sialoglycoprotein endopeptidase digestion. Transient overexpression of endomucin-1/-2 reduced the number of adhesion plaques and reduced cell attachment to the substrate. This phenotype was suppressed by laminin or the protein kinase inhibitor staurosporine. Our findings suggest that human endomucin-1/-2 negatively regulate cell adhesion to the extracellular matrix.

Key words: Signal sequence trap; Sialomucin; Vascular endothelium; Anti-adhesion

1. Introduction

Cell–extracellular matrix (ECM) interaction in many types of cells is mediated by an integrin-based submembranous assembly of proteins termed the focal adhesion complex or adhesion plaque [1]. Formation of focal adhesion complexes is regulated by cascades of protein–protein interaction and protein phosphorylation [2]. Several extracellular macromolecules are known to interfere with cell–ECM interaction; such molecules include proteoglycans, glycoproteins, and sialoglycoproteins [3]. Mucin-like sialoglycoproteins are known to interfere with cadherin-mediated cell–cell interaction as well as integrin-mediated cell–ECM interaction [4–6]. Steric hindrance by their long extracellular domains is postulated as the mechanism underlying such interference. For instance, MUC-1/episialin has sialoglycosylated extracellular domains of 1000–2200 amino acid residues spanning a distance as great as 200–500 nm.

Several lines of evidence suggest that cell adhesion may also be regulated in a negative fashion by intracellular mechanisms. For instance, an intracellular protein phosphatase, PTEN, suppresses integrin-mediated cell spreading and focal adhesion assembly, probably by antagonizing the focal adhesion kinase FAK [7]. Another intracellular protein, phosphoinositide 3-kinase, is essential for cell detachment by thrombospondin, an anti-adhesive extracellular glycoprotein [8]: phosphatidylinositol-3,4,5-trisphosphate, a product of the reactions catalyzed by phosphoinositide 3-kinase, can rearrange focal adhesion complexes by interfering with the interaction between integrin and α-actinin [9].

In a cDNA screen designed to isolate cell surface molecules involved in mammalian brain development, we identified a family of type I membrane proteins termed endomucin-1 and -2. A mouse homolog of endomucin-1 has recently been reported to be expressed on the vascular endothelial cell surface, although its function has not been elucidated [10]. Here we report that both human endomucin-1 and -2 interfere with the assembly of focal adhesion complexes and inhibit cell–ECM interaction.

2. Materials and methods

2.1. Library construction and signal sequence trap screening

A cDNA expression library was constructed using poly(A)$^+$ RNA extracted from mouse brains at gestational day 14 as described previously [11–14]. In brief, cDNA fragments generated using 5 μg poly(A)$^+$ RNA and random primers were size-fractionated at 0.4–0.8 kb and ligated uni-directionally into a yeast expression vector, pSuc2t7-F1ori, designed to express cDNA-invertase fusion transcripts. This enzyme cannot be secreted from cells unless the cDNA encodes an in-frame signal peptide. After transformation of the *Saccharomyces cerevisiae* strain YTK12, transformants (4×10^5 independent clones) were selected with trisaccharide-based media to isolate clones producing secretable invertase. cDNAs were amplified out of the yeast genomic DNA by PCR (polymerase chain reaction) and sequenced using a DNA sequencer (ABI373; Perkin Elmer/ABI, Foster City, CA, USA).

2.2. Isolation of endomucin-2 cDNA

We performed 5′-RACE (rapid amplification of the 5′-cDNA end) for *endomucin-2* using a human heart-derived cDNA pool (Marathon-Ready, Clontech, Palo Alto, CA, USA), a universal primer supplied by the manufacturer, and a specific primer, 5′-CCTCAGTGCCTATTACTTGAGACTGTGAGG-3′ (complementary strand

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[1] The nucleotide sequences reported in this paper are available via GenBank accession numbers AB034694 (mouse *endomucin-1*), AB034695 (human *endomucin-1*), and AB034696 (human *endomucin-2*).

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of AA426230, bases 13–42). After 35 cycles of PCR, a single 0.7 kb product was obtained and cloned into pBluescript SK- (Stratagene, La Jolla, CA, USA), and the nucleotide sequence was determined with the DNA sequencer.

2.3. RNA blot hybridization

Mouse and human MTN Blots (Clontech) were hybridized with ^{32}P-labeled mouse *endomucin-1* cDNA (template: clone AA111512) and washed with 0.1×SSC, 0.1% SDS at 60°C. Signals were detected using BAS2000 (Fujix, Tokyo, Japan). The same membranes were reprobed for human *endomucin-1* and *-2*.

2.4. Construction of expression vectors

To construct vectors expressing human *endomucin-1* and *-2* cDNAs, PCR-amplified human cDNAs were digested with *Eco*RI plus *Bam*HI and ligated between the *Eco*RI and *Bgl*II sites of a mammalian expression plasmid, pCAGGS [15]. A PCR-generated DNA fragment encoding endomucin-1ΔSS, which lacks the NH_2-terminal signal sequence (amino acid residues 2–20), was also ligated into pCAGGS. To express green fluorescent protein (GFP) fusion proteins, PCR-generated human *endomucin-1/-2* cDNAs lacking the termination codon were ligated respectively into the *Hin*dIII site and between the *Xho*I–*Bam*HI sites of pEGFP-N3 (Clontech). To express FLAG-tagged human endomucin-1/-2, PCR-generated DNA fragments for endomucin-1ΔSS or -2ΔSS were inserted into the *Hin*dIII site or between the *Eco*RI–*Bam*HI sites of the pFLAG-CMV1 vector (Kodak/IBI, New Haven, CT, USA). The resulting plasmids express FLAG-tagged endomucin-1ΔSS/-2ΔSS containing the vector-derived, preprotrypsin signal sequence at their NH_2-termini. We confirmed that these FLAG-tagged proteins were properly targeted to the plasma membrane in HeLa cells by immunofluorescence staining with the anti-FLAG antibody M2 (Sigma, St. Louis, MO, USA) (data not shown). The intactness of each cDNA generated by PCR was confirmed by sequencing.

2.5. Cell culture and transfection

NIH3T3 (mouse fibroblast), HeLa (derived from human uterine cancer), and HEK293T (derived from human embryonic kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in glass-bottom dishes (MatTek, Ashland, MA, USA). Plasmids were transfected with a calcium phosphate-based transfection reagent (CellPhect, Amersham Pharmacia Biotech, Piscataway, NJ, USA) 24–48 h before observation or protein extraction. For microscopic identification of cells expressing untagged endomucin transgenes, pEGFP-N3 was co-transfected at a molar ratio of 10:1 (endomucin: GFP marker).

2.6. Protein analyses

HEK293T cells (~10^7) transfected with the expression vectors for FLAG epitope-tagged human endomucin-1 or -2 were lysed 24 h after transfection with 2 ml lysis buffer (20 mM Tris–HCl pH 7.4, 0.1% SDS, 1% Triton X-100, 1 mM PMSF, 1 U/ml aprotinin). The samples were cleared by centrifugation at 20 000×*g* for 30 min, and the supernatant was incubated at 4°C for 1 h with 10 μl agarose beads conjugated with anti-FLAG antibodies (Kodak/IBI). The beads were washed twice with 1 ml lysis buffer and resuspended in 50 μl Tris-buffered saline (TBS; pH 7.4) with or without 24 μg *O*-sialoglycoprotein endopeptidase (OSGE; Cederlane Labs, ON, Canada). After incubation at 0°C or 37°C for 2 h, the beads were washed twice with 1ml PBS, and the proteins were heat-denatured in a reducing SDS buffer, resolved by SDS–PAGE (10% acrylamide), and transferred onto a nitrocellulose membrane. The membrane was pretreated with TBS containing 4% skimmed milk and 0.1% Tween 20 (TBST), incubated with the anti-FLAG antibody, washed three times with TBST, and incubated with alkaline phosphatase-conjugated anti-mouse IgG (Promega). After washing with TBST, a chromogenic reaction was performed with a stabilized substrate for alkaline phosphatase (Promega) for 10 min.

2.7. Immunofluorescence microscopy

Mouse monoclonal antibodies against vinculin (VIN-11–5, Sigma) and phosphotyrosine (Upstate Biotechnology, Lake Placid, NY, USA) were used at a concentration of 1 μg/ml as primary antibodies. Methods for immunofluorescence staining and fluorescence microscopy were as described previously [16–18].

3. Results

3.1. Isolation of human and mouse endomucin-1 cDNAs

Mouse embryonic cDNAs encoding extracellular proteins were screened by the signal sequence trap method using *S. cerevisiae* as a host [11–14]. This method selects yeast transformants that secrete signal sequence/invertase fusion proteins and thus are able to utilize trisaccharides as a carbon source.

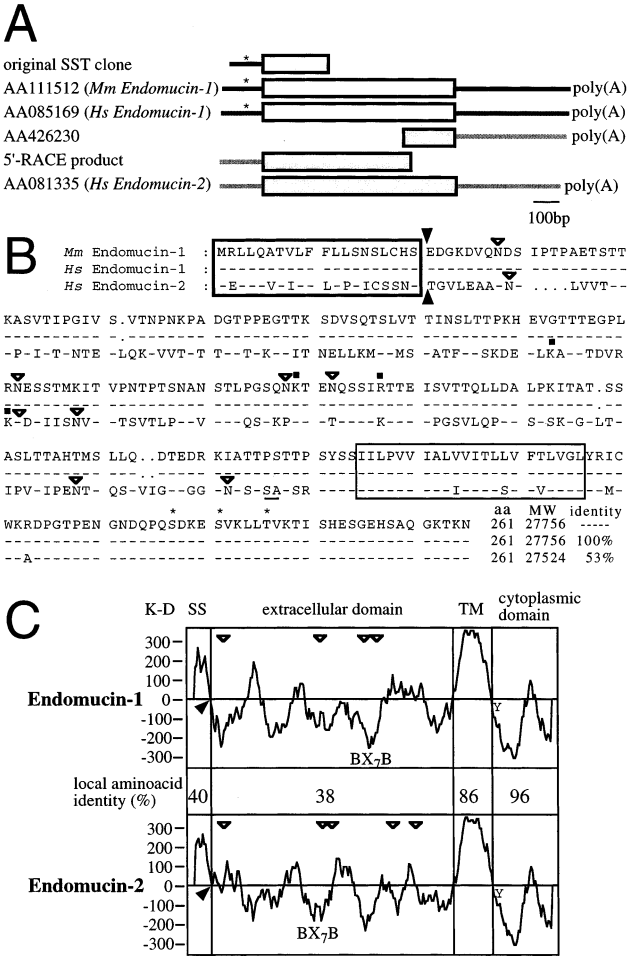

Fig. 1. Structure of the human and mouse *endomucin-1* and *-2* cDNAs and their products. A: A schematic representation of the cDNA fragments analyzed in this study. The open boxes represent ORFs, with or without preceding in-frame stop codons (*). B: Alignment of the deduced amino acid sequences of mouse endomucin-1 and human endomucin-1 and -2. A dash and a dot respectively represent an identical amino acid residue and a gap inserted for maximal alignment. The size (in amino acid residues and molecular weight) and the overall amino acid identity (%) to mouse endomucin-1 are shown after each sequence. The putative signal sequences and the transmembrane domains are boxed with bold and thin lines, respectively. Potential *N*-glycosylation sites are indicated by triangles. The basic residues in BX7B motifs are indicated by black squares. Possible sites for glycosaminoglycation are underlined. Potential phosphorylation sites for protein kinase C are indicated by asterisks. C: Hydrophobicity profiles of human endomucin-1 and -2 based on the Kyte–Doolittle (K–D) method. Higher K–D scores indicate higher hydrophobicity. The putative cleavage sites after the signal sequences (SS) are indicated by arrowheads. Putative *N*-glycosylation sites (triangles) and the BX7B motifs in the extracellular domains (BX7B) are also denoted. The local amino acid identities (in %) are shown between the two profiles.

Two hundred and ninety two clones out of 1.4×10^5 transformants survived this selection, and the cDNAs from these clones were sequenced. A database search revealed that these clones included a number of known genes encoding secreted or membrane-bound proteins as well as unknown genes encoding proteins with putative signal sequences. Among these, 12 independent clones were found to encode a common polypeptide. A search of the GenBank database found two matching EST (expressed sequence tag) sequences: one from mouse (GenBank accession number AA111512) and one from human (AA085169). We obtained and sequenced the two EST clones and found that our clone corresponded to the 5′-terminal portion of the EST clones (Fig. 1A). Each EST clone contained an open reading frame (ORF) of 261 amino acid residues with identical amino acid sequences (Fig. 1B). The coding regions of the mouse and human ESTs are 99.9% conserved at the nucleotide sequence level. While this study was in progress, the mouse endomucin cDNA sequence, which was virtually identical to the mouse EST clone we analyzed except for a deletion of 39 bases corresponding to amino acids T129–S141, was reported [10]. Since all the mouse and human ESTs in the current database are without this deletion, the longer cDNA may represent the major mRNA species. In this report, we refer to the longer mouse cDNA and its human counterpart as *endomucin-1* to distinguish these from the shorter cDNA species in the mouse [10].

3.2. Isolation of the human endomucin-2 cDNA

A database search with the putative endomucin-1 peptide sequence detected another human EST clone (AA426230) which appeared to be truncated at the 5′-end (Fig. 1A). We performed 5′-RACE based on the sequence of this clone using a human cDNA pool and obtained a single cDNA of ~700 bp (see Section 2). Sequence analysis of the cDNA revealed its identity with another EST clone (AA081335; partially sequenced). We obtained this EST clone, and DNA sequencing revealed that it contained an ORF of 261 amino acid residues preceded by a consensus for translational initiation [19] and followed by a polyadenylation signal. Since 5′-RACE gave no clone that extended beyond the 5′-end of AA081335, this clone probably contains the entire ORF. As the encoded polypeptide is similar to, but distinct from, the product of the *endomucin-1* cDNA (see below), we named this cDNA human *endomucin-2*.

3.3. Structures of endomucin-1 and -2

The mouse *endomucin-1* gene and the human *endomucin-1* and *-2* genes encode polypeptides of 261 amino acid residues with calculated molecular masses of 27.8, 27.8, and 27.5 kDa, respectively. Hydrophobicity profiles [20] predicted that these polypeptides contain two major hydrophobic stretches (Fig. 1C). In each polypeptide, the NH_2-terminal hydrophobic stretch of 20 residues is followed by a putative cleavage site for signal peptidases [21]. The second hydrophobic stretch is likely to be a transmembrane domain and is flanked by two hydrophilic regions probably representing extracellular and cytoplasmic domains. Thus, both endomucin-1 and -2 polypeptides are likely to be processed to yield type I membrane proteins of 241 amino acid residues.

The overall amino acid identity between endomucin-1 and -2 is 53%. Their similarity is highest in the putative cytoplasmic (96%) and transmembrane (86%) domains, and more diverged in the signal sequences (40%) and the extracellular domains (38%) (Fig. 1C). The extracellular domains are rich in three hydrophilic amino acids, serine, threonine, and proline, which account for 43–45% of the amino acid residues in this domain. Many of these serine and threonine residues are potential *O*-glycosylation sites; by the algorithm of Hansen et al. [22], 15 sites in endomucin-1 and 14 sites in endomucin-2 are predicted to have a probability of >0.9 of being *O*-glycosylated. In addition, each polypeptide has a consensus sequence for glycosaminoglycan attachment: one of the hydrophilic troughs in each extracellular domain contains a heparin binding motif, BX_7B [23]. Unlike the typical mucin core polypeptides, however, endomucin-1 and -2 have no MUC-1 repeat in their extracellular domains [24]. In the conserved cytoplasmic domain, each endomucin contains a sequence which is conserved in the rhodopsin-like G protein-coupled receptor superfamily (residues 186–207; searched at http://motif.genome.ad.jp) and three potential phosphorylation sites for protein kinase C (http://www.cbs.dtu.dk/databases/PhosphoBase/). Thus, endomucin-1/-2 may function as cell surface receptors which act to transduce extracellular signals.

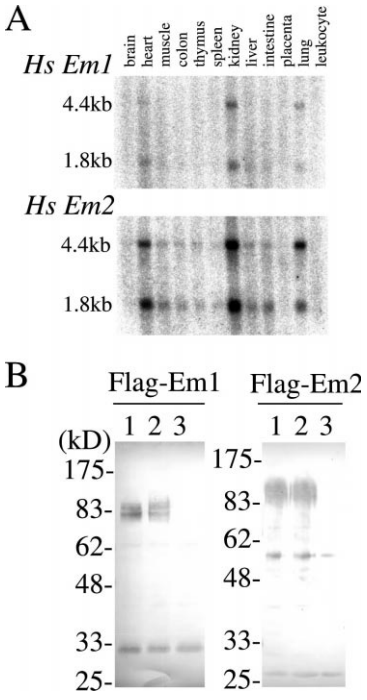

Fig. 2. Endomucin mRNAs and proteins. A: Expression of the *endomucin-1* and *-2* genes (abbreviated as Em1 and Em2) in various human tissues were detected by RNA blot hybridization. Each lane contained 1 μg of poly(A)$^+$ RNA extracted from the indicated tissues. B: FLAG-endomucin-1 and FLAG-endomucin-2 transiently expressed in HEK293T cells were immunoprecipitated with anti-FLAG antibodies, and then incubated for 2 h either on ice (lane 1) or at 37°C (lanes 2 and 3) without (lanes 1 and 2) or with OSGE (lane 3). The tagged proteins were detected by immunoblot assay using anti-FLAG antibodies. Note that the majority of the tagged endomucin-1 and -2 were post-translationally modified and ran at an apparent size of 80–110 and 90–120 kDa, respectively, and were completely degraded by OSGE treatment. A minor band in the FLAG-endomucin-2 blot (~55 kDa) that showed partial susceptibility to OSGE may represent an *O*-sialoglycosylation intermediate. The other minor bands of <30 kDa were resistant to OSGE, suggesting that they may represent unmodified core proteins. Their resistance to OSGE argues against non-selective degradation of the major bands by contaminating proteases.

3.4. Expression of the endogenous endomucin-1 and -2 genes

Expression patterns of *endomucin-1* and *-2* in human tissues were assessed by RNA blot hybridization (Fig. 2A,B). Both genes are expressed in many organs, and the signals are especially strong in the heart, kidney, and lung. The *endomucin-2* probe consistently yielded stronger signals than the *endomucin-1* probe under similar experimental conditions, suggesting higher steady-state expression of the *endomucin-2* gene in human tissues. Both probes detected two mRNA species; the lengths of the EST clones (~1.5 kb without poly A tail) are comparable to the shorter transcripts (~1.8 kb). Although the identities of the longer transcripts (~4.4 kb) are currently unknown, the concordant expression patterns of the two transcripts suggest that they may represent alternatively processed mRNA species transcribed from the same gene. Mouse tissue blots probed with the human *endomucin-1* cDNA showed abundant expression of the ~1.8 kb transcript in the heart, kidney, and lung (data not shown), which is consistent with the data in a previous report [10]. Interestingly, however, the human *endomucin-2* probe detected no signals on the mouse RNA blots (data not shown). These results, together with the fact that no *endomucin-1*-related genes were isolated in our initial screening, suggest that the mouse genome may not contain the orthologue of the human *endomucin-2* gene, or even if it does, the gene may be highly diverged or expressed at only very low levels.

3.5. Subcellular localization and O-sialoglycosylation of the endomucin-1 and -2 proteins

Subcellular localization of human *endomucin-1* and *-2* was assessed by expressing these proteins tagged with the *Aequorea* GFP at their carboxy-termini. When transiently expressed in HEK293T and HeLa cells, the endomucin-1–GFP and endomucin-2–GFP consistently localized to the plasma membrane and less abundantly to Golgi-like perinuclear stacks (Fig. 3E–G,J and data not shown). To analyze post-translational modification, endomucin-1 and -2 tagged with the FLAG epitope (see Section 2) were expressed in HEK293T cells. Using immunoprecipitation and immunoblot detection with anti-FLAG antibodies, FLAG-endomucin-1 appeared as two bands, a major broad band ranging from 80 to 100 kDa and a minor band around 30 kDa. FLAG-endomucin-2 appeared as three bands, a major broad band ranging from 90 to 120 kDa, a minor band around 60 kDa, and a second minor band a little under 30 kDa (Fig. 2B, lanes 1). FLAG-endomucin products were not detected in the tissue culture media 48 h after transfection (data not shown), suggesting that secretion or shedding of the extracellular domain is unlikely, at least in this system.

Since extracellular domains of endomucin-1 and -2 have numerous potential *O*-sialoglycosylation sites, we tested the possibility of *O*-sialoglycosylation by treating the immunoprecipitated proteins with *Mannheimia O*-sialoglycoprotein endopeptidase which specifically cleaves *O*-sialoglycosylated proteins [25]. Both of the major high molecular weight FLAG-endomucin bands completely disappeared after OSGE-treatment (Fig. 2B, lanes 3), demonstrating susceptibility of these proteins to OSGE due to *O*-sialoglycosylation. Taken together, these results indicate that endomucin-1 and -2 polypeptides are *O*-sialoglycosylated, targeted to the plasma membrane, and retained there as type-I membrane proteins with apparent molecular weights of 80–100 and 90–120 kDa, respectively.

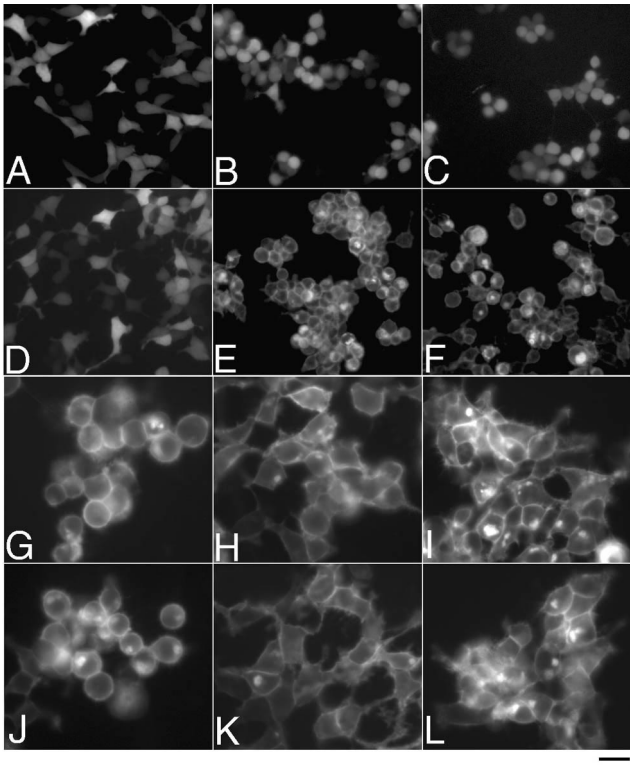

Fig. 3. Biological activity and subcellular localization of endomucin-1 and -2 expressed in the human cell line HEK293T. Cells were co-transfected with a GFP-expression vector (transfection marker) plus the control vector (A), or the vector expressing endomucin-1 (B), endomucin-2 (C), or endomucin-1ΔSS (D). Most of the endomucin-1/-2 transfectants with green fluorescence (shown as white signals) were round-shaped at 24 h after transfection (B and C). In contrast, the control vector and the vector expressing endomucin-1 without a signal sequence (ΔSS) showed no such activity (A and D). Endomucin-1–GFP (E) and endomucin-2–GFP (F) fusion proteins were concentrated on the plasma membrane and in Golgi-like structures, and exhibited similar rounding inducing activity as the untagged proteins. Higher magnification of endomucin-1–GFP (G–I) and endomucin-2–GFP expressing cells (J–L) at 48 h after transfection. Some cells are detached from the uncoated glass surface and out of focus (G and J). Coating of the glass surface with 1 μg/cm^2 laminin (H and K) or addition of 10 nM staurosporine to the medium (I and L) suppressed the morphological changes in spite of the persistent membrane localization of endomucin–GFPs. Bar: 20 μm in A–F, 40 μm in G–L.

3.6. Overexpression of endomucin-1 or -2 promotes cell detachment

To study the biological activities of the endomucins, we co-transfected a vector expressing the untagged human endomucin-1 or -2 and a GFP-expression vector into HEK293T cells and observed the morphology of the transfected cells. Interestingly, 24–48 h after transfection the majority of the transfectants had become round-shaped and some of them were detached from the glass surface (Fig. 3B,C). The GFP-fused endomucins also induced similar morphological changes in HEK293T cells (Fig. 3E–G,J). In contrast, endomucin-1ΔSS, lacking the signal sequence, failed to show such activity (Fig. 3D and data not shown), suggesting that membrane localization is essential for this activity. We also tested the importance of the putative hyaluronate binding motifs (BX_7B) in the extracellular domain of endomucin-1 by replacing one of the

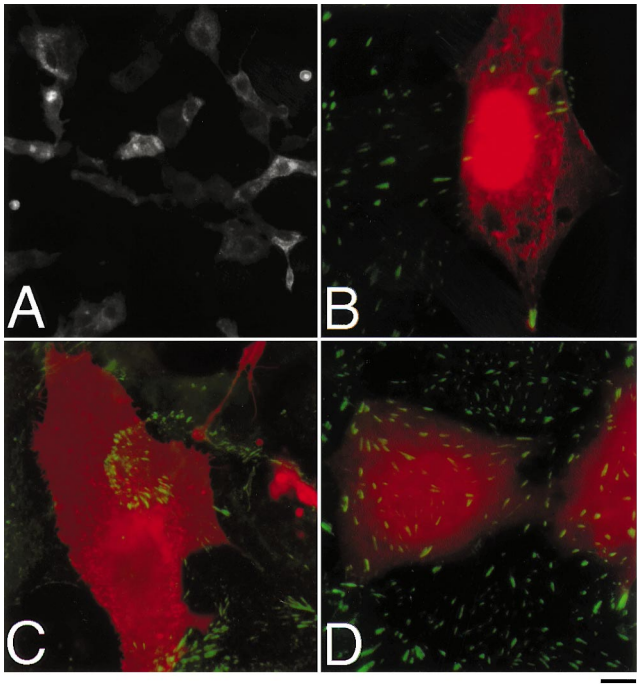

Fig. 4. Endomucin-1 overexpression interferes with assembly of focal adhesion complex in fibroblasts. A: NIH3T3 cells 24 h after transfection of endomucin-1–GFP exhibited minimal morphological changes except a few rounded cells. Subcellular localization of the fusion protein was comparable to that observed in human epithelial cell lines. B–D: Effects of endomucin-1 expression on focal adhesion assembly. Pseudocolor images of fixed transfectants expressing endomucin-1–GFP (B, C) or GFP alone (D) with immunostaining either for vinculin (red) or phosphotyrosine (green). Note that untransfected cells with numerous focal adhesion complexes are underlying the transfected cells, and that these transfected cells have poorly organized focal adhesion complexes (B and C). Bar: 20 μm in A, 120 μm in B–D.

two basic residues in these motifs with alanine (i.e. K128A and R136A). These mutants retained the rounding/detachment inducing activity (data not shown), suggesting that these motifs have little or no role in this particular activity.

3.7. *Overexpression of endomucin-1 interferes with the assembly of focal adhesion complex*

To test the possible functional interaction between endomucins and the focal adhesion complex, we expressed endomucin-1–GFP in NIH3T3 cells, whose adhesion mechanism has been well studied. Unlike HEK293 and HeLa cells, NIH3T3 cells did not show any obvious rounding or detachment after endomucin-1–GFP expression (Fig. 4A and data not shown). Interestingly, however, the number of adhesion plaques labeled with antibodies against vinculin or phosphotyrosine were markedly reduced (Fig. 4B,C) in comparison with the cells transfected with the control GFP-expression vector (Fig. 4D). Moreover, untransfected cells with numerous adhesion plaques (Fig. 4B,C; cells without red pseudocolor) were frequently seen beneath the transfectants, suggesting a loss of tight contact between the endomucin expressing cells and the glass surface. These observations indicate that overexpression of endomucin-1 interfered with assembly of focal adhesion complexes in fibroblasts, causing partial detachment of the cells from the substrate.

3.8. *Anti-adhesive activity of endomucin-1/-2 is suppressed by ECM proteins and staurosporine*

To find clues to the mechanism by which endomucin affects focal adhesion complexes and cell attachment, we tested the effects of culture substrate (extracellular signals) and protein kinase inhibitors (intracellular signals) on the anti-adhesive activity of endomucins. When HEK293T cells expressing endomucin-1–GFP or endomucin-2–GFP were plated on a laminin-coated glass surface, they remained attached with a flat morphology (Fig. 3H,K). Similar effects were observed with other ECM proteins such as collagen-I and fibronectin (data not shown). We also found that a protein kinase inhibitor, staurosporine, promoted attachment of these endomucin expressing cells to the uncoated glass surface (Fig. 3I,L). Staurosporine is known to promote cell spreading by modifying focal adhesion components [26]. Although increased cytoplasmic accumulation of the endomucin–GFP fusion proteins was seen, a significant proportion of the endomucin–GFP signal was found on the plasma membrane. Thus, the anti-adhesive activities of endomucin-1 and -2 are reversible by ECM proteins or a protein kinase inhibitor that are known to promote cell adhesion. These findings suggest that the rounding and detachment of the cells overexpressing endomucin-1/-2 reflect an interference with the cell adhesion machinery rather than non-specific cytotoxicity by protein overproduction.

4. Discussion

We have isolated and characterized a series of mammalian cDNAs encoding structurally related membrane-bound *O*-sialoglycoproteins, endomucin-1 and -2. We also showed that they are widely expressed in human tissues and have the potential to interfere with the assembly of focal adhesion complexes and cell attachment. Among the mucin-like sialoglycoproteins characterized to date, endomucin-1/-2 are unique in their short extracellular domains (170 amino acid residues) and the lack of MUC-1 repeats. Thus, whether endomucin-1/-2 exert their anti-adhesive activity through a mechanism distinct from that used by other mucin-like proteins is an important question that remains to be addressed in future studies.

Our finding that ECM proteins or staurosporine can suppress the anti-adhesive activity of endomucin-1/-2 suggests the involvement of some intracellular signaling events, rather than mere physical interference of cell–ECM interaction, in this activity. Staurosporine is known to promote integrin-mediated cell adhesion by stimulating tyrosine phosphorylation of Src and tensin in the focal adhesion complex [26]. Additionally, capping of sialomucins, CD34 and CD43, is known to activate Syk and Lyn tyrosine kinases in hematopoietic cells [27]. Thus, endomucin-1/-2 might also alter cell behavior through protein kinases that regulate the assembly of focal adhesion complexes. Identification of cellular proteins interacting with the highly conserved cytoplasmic domains of endomucin-1 and -2 may provide clues for the elucidation of such intracellular events.

We attempted to raise specific antibodies against endomucin-1 and -2 using six distinct synthetic oligopeptides as antigens. Unfortunately, however, none of the sera recognized mature proteins in mouse tissues or recombinant proteins expressed in cultured cells. This is probably because the endomucin core peptides are highly deformed and/or masked by

the sialoglycoside moieties. This is also consistent with previous findings that antibodies against mouse endomucin were less reactive toward the deglycosylated protein [10].

Mouse endomucin was found to be expressed on vascular endothelial cells [10], and this led to the proposal that endomucin may function as a negative regulator for endothelial attachment to the basement membrane or cell–cell adhesion among endothelial cells. Another possible role for endomucins is negative regulation of leukocyte adhesion onto the vascular endothelia. In support of this hypothesis, mouse endomucin is not expressed on the luminal surface of high endothelial venule (HEV) [10], a specialized site for leukocyte attachment in lymphoid organs. Rather, another sialoglycoprotein, CD34, expressed on HEV serves as a ligand for a leukocyte adhesion molecule, L-selectin [28]. This raises a third possibility, that endomucins might function as ligands, receptors, or co-receptors for cell adhesion and/or cell recognition systems. Interestingly, CD43 is known to be differentially sialoglycosylated to yield 130 kDa and 115 kDa molecules with different anti-adhesive activity [29]. Likewise, the epitope-tagged endomucin-1 and -2 expressed in cultured cells (80–100 kDa and 90–120 kDa, respectively) as well as the mouse endogenous endomucin of 70–90 kDa [10] may represent molecules with variable *O*-silaloglycosylation patterns and distinct activities. It may therefore be important to focus on the biologically relevant glycoforms in future functional studies.

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