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CD43 has a functional NLS, interacts with β-catenin, and affects gene expression

Christian X. Andersson,[a] Julia Fernandez-Rodriguez,[a] Sirle Laos,[a] Rein Sikut,[b] Anu Sikut,[b] Dan Baeckström,[a] and Gunnar C. Hansson[a,*]

[a] *Department of Medical Biochemistry, Göteborg University, Medicinaregatan 9A, 413 90 Gothenburg, Sweden*
[b] *Institute of Molecular and Cell Biology, University of Tartu, 23 Riia Str., 51010 Tartu, Estonia*

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Abstract

CD43 is a transmembrane molecule with a highly O-glycosylated extracellular domain of mucin type. It is a normal constituent of leukocytes and found in colon adenoma, but not in normal colon epithelia. Here it is shown that the cytoplasmic tail of CD43 contains a functional bipartite nuclear localization signal directing it to the nucleus. The intracellular domain of CD43 interacts with β-catenin and causes an upregulation of the β-catenin target genes *c-MYC* and *CyclinD1*. The present results suggest that CD43 can be involved in nuclear signaling and via β-catenin interaction be involved in cell proliferation.

Keywords: CD43; beta-Catenin; Mucin; Nucleus; NLS

A number of membrane anchored single-span glycoproteins with a highly glycosylated extracellular domain have been implied in cell interaction phenomena. The extracellular domains of these are characterized by the amino acids Ser, Thr, and Pro and their extensive O-glycosylation classifies these to the mucin family. The transmembrane domain is followed by a cytoplasmic C-terminus containing different motifs known to be involved in signaling. The extensive and variable glycosylation on the extracellular domain and lack of typical receptor domains have made it difficult to understand their way of function and how signals are transmitted. CD43 (leukosialin, sialophorin), the major cell surface molecule in most hematopoietic cells, is an example of such a mucin-type molecule. It has an extracellular domain consisting of 235 amino acid that extends 45 nm from the cell surface and carries abundant O-glycans [1]. The transmembrane domain is 23 amino acid long followed by a 123 amino acid cytoplasmic tail (ct), containing several Ser/Thr phosphorylation sites [2]. CD43 is suggested to have both adhesive and anti-adhesive properties in leukocytes [3]. An example of the latter is its exclusion from the immunological synapse of the T-cell allowing the T-cell receptors to approach the antigen-presenting cell. This rearrangement of CD43 is directed by the cytoskeleton via complexes formed with Ezrin (an ERM protein) and CD43 cytoplasmic tail, CD43ct [4]. CD43 is also involved in T-cell activation as suggested by experiments where CD43 antibody cross-linking leads to Fyn and Vav tyrosine phosphorylation probably via binding to the SH3 binding domain of CD43ct [5,6]. It is evident that CD43 has important regulatory functions in leukocytes.

In addition to hematopoietic cells, CD43 has been found in different cancer cells, colon adenomas, and in some colon carcinomas, both as protein and mRNA [7,8]. This prompted us to further study the function of CD43 outside of hematopoietic cell lineages. In this report, CD43ct is shown to contain a functional nuclear localization signal (NLS), to interact with β-catenin, and cause activation of *c-MYC* and *CyclinD1* genes.

* Corresponding author. Fax: +46-31-416108.
E-mail address: gunnar.hansson@medkem.gu.se (G.C. Hansson).

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Materials and methods

Cells and antibodies. COLO 205 (CCL-222), SW480 (CCL-228), and CHO-K1 (CCL-61) cells were cultivated in Iscove's medium containing 10% fetal bovine serum and for transfections DMRIE-C (Invitrogen) was used. The mAb against CD43, αCD43-3A1, αCD43-4D2, and αCD43-L10 has previously been described [8,9] and the MYC 9E10.2 hybridomas were obtained from ATCC (CRL-1729). All other antibodies were obtained from BD. Dilutions were 1:500 for Western blot, 1:200 for FACS and 5 μg was used for immunoprecipitation. Nuclear and cytoplasmic lysates were prepared by the NE-PER reagents (Pierce). Cells were fixed and stained [8] with the αCD43-3A1 mAb diluted to 10 μg/ml. Nuclei were stained with propidium iodide (2 μg/ml) for 5 min. After staining, coverslips were mounted with Pro-Long antifade (Molecular probes). Confocal images were collected with a Leica Confocal Microscope (TCS NT/SP Series) using a 63× oil immersion objective.

Expression plasmids. The intracellular domain of CD43 was cloned into the expression vector pEGFP-C1 (BD-Clontech) giving the vector pGFP-CD43ct. Point mutations were made by QuikChange Site-Directed Mutagenesis Kit (Stratagene) generating the mutants GFP-CD43ct(NLS1), GFP-CD43ct(NLS2), and GFP-CD43ct(NLS1 + 2). The pGST-CD43ct plasmid was constructed by cloning in CD43ct into a pGEX-4T-1 vector (Amersham Bioscience). All constructs were sequenced.

Immunoprecipitations, pull-downs, and Western blot. Immunoprecipitations were performed using Dynabeads M-450 goat-α mouse IgG (Dynal) or protein G PLUS–agarose (Santa Cruz). The samples were separated by SDS–PAGE, transferred electrophoretically onto Immobilon P (Millipore), and probed with antibodies [8,9]. For pull-down experiments the GST-fusion proteins were attached to glutathione Sepharose 4B (Amersham Bioscience) and incubated in cell lysate for 14 h at 4 °C followed by thoroughly washing in lysate buffer.

Reporter system. The reporter system, pTOPFLASH and pFOPFLASH [10], was transfected into SW480 cells stably expressing either GFP or GFP-CD43ct. Co-transfection with the *Renilla* luciferase vector was used as internal control and Dual-Glo Luciferase Assay System and *Renilla* Luciferase Assay System (Promega) for measuring the luciferase activity 24 h after transfection. Protein amount, measured by BCA assay (Pierce), and *Renilla* luciferase activity were compensated for when calculating the relative luciferase activities.

Flow cytometry. Cells were washed in PBS, fixed in 4% paraformaldehyde at RT for 15 min, permeabilized in 0.1% saponin solution on ice for 30 min, and incubated with primary antibodies on ice for 30 min. Biotin conjugated goat-α mouse secondary antibody (Dako) was used (1:200) and incubated on ice for 30 min followed by allophycocyanin–streptavidin substrate (Perkin–Elmer) (1:200). The cells were analyzed with a FACSCalibur cell sorter (BD).

Results

Nuclear localization signal in the cytoplasmic tail of CD43

When analyzing the sequence of the CD43ct, a potential nuclear localization signal (NLS) was found close to the transmembrane domain. This NLS is following the rules for a bipartite signal and consists of two clusters of positively charged amino acids separated by 10 amino acids (KRR-10X-KR) and located at the amino acid position 282–296 (Fig. 1A). The CD43ct was expressed as a fusion protein with the EGFP-tag at its N-terminal (Fig. 1A). The colon carcinoma cell line SW480 and CHO cells were transfected with these constructs and analyzed by confocal microscopy showing that GFP-CD43ct localized to the nucleus, whereas GFP alone was found throughout the cells (Figs. 1B and C).

To address whether the NLS was functional or not, a mutagenesis study was performed in the pGFP-CD43ct vector. The Lys and Arg in the two basic amino acid clusters were separately mutated to Pro and Gly, respectively. The resulting plasmids pGFP-CD43ct(NLS1) and pGFP-CD43ct(NLS2) were transfected into SW480 cells and their localization was studied (Fig. 1B). Both NLS-mutants still showed nuclear staining although not as distinct as for the non-mutated GFP-CD43ct. However, when both clusters were mutated at the same time (pGFP-CD43ct(NLS1 + 2)), the fluorescence in transfected cells was essentially localized to the cytoplasm.

To address if endogenous CD43 could be localized to the nucleus, the CD43-expressing colon carcinoma cell line COLO 205 was stained with the αCD43-3A1 mAb which reacts with CD43ct. Confocal microscopy showed a nuclear staining in subconfluent cells as compared to confluent cells where the staining was concentrated to the cell membrane, typical for CD43 in leukocytes (Fig. 1D). This suggests that CD43 can localize to the nucleus.

Interaction between CD43 and β-catenin

As CD43ct contains a signal for translocation to the cell nucleus, we asked if CD43ct had any functional role in the nucleus related to CD43 expression in colon cancer and adenoma. In most colon cancers, β-catenin is translocated into the nucleus where it regulates specific gene expression important for cancer development [11]. To test for possible interaction between CD43 and β-catenin, immunoprecipitations of CD43 using the mAbs αCD43-3A1, αCD43-4D2 (both reactive with CD43ct), and αCD43-L10 (binding the glycosylated extracellular domain) were made in lysates from the CD43-expressing colon carcinoma cell line COLO 205 followed by Western blotting using a β-catenin mAb. β-Catenin was found to be co-precipitated with CD43 by all three antibodies, but not with normal mouse serum (Fig. 2A). The association of β-catenin with CD43 as shown by the αCD43-L10 mAb suggests that this interaction can take place at the cell membrane. The CD43/β-catenin interaction could also occur in the nucleus, as immunoprecipitations with the mAb αCD43-3A1 in nuclear lysates from COLO 205 cells could co-precipitate β-catenin (Fig. 2B). The CD43ct appeared to be sufficient for binding β-catenin, as a recombinant GST fusion protein with the CD43ct could pull down β-catenin in the COLO 205 cell lysate, whereas the GST protein alone did not (Fig. 2C). The GFP-CD43ct fusion protein that localized to the

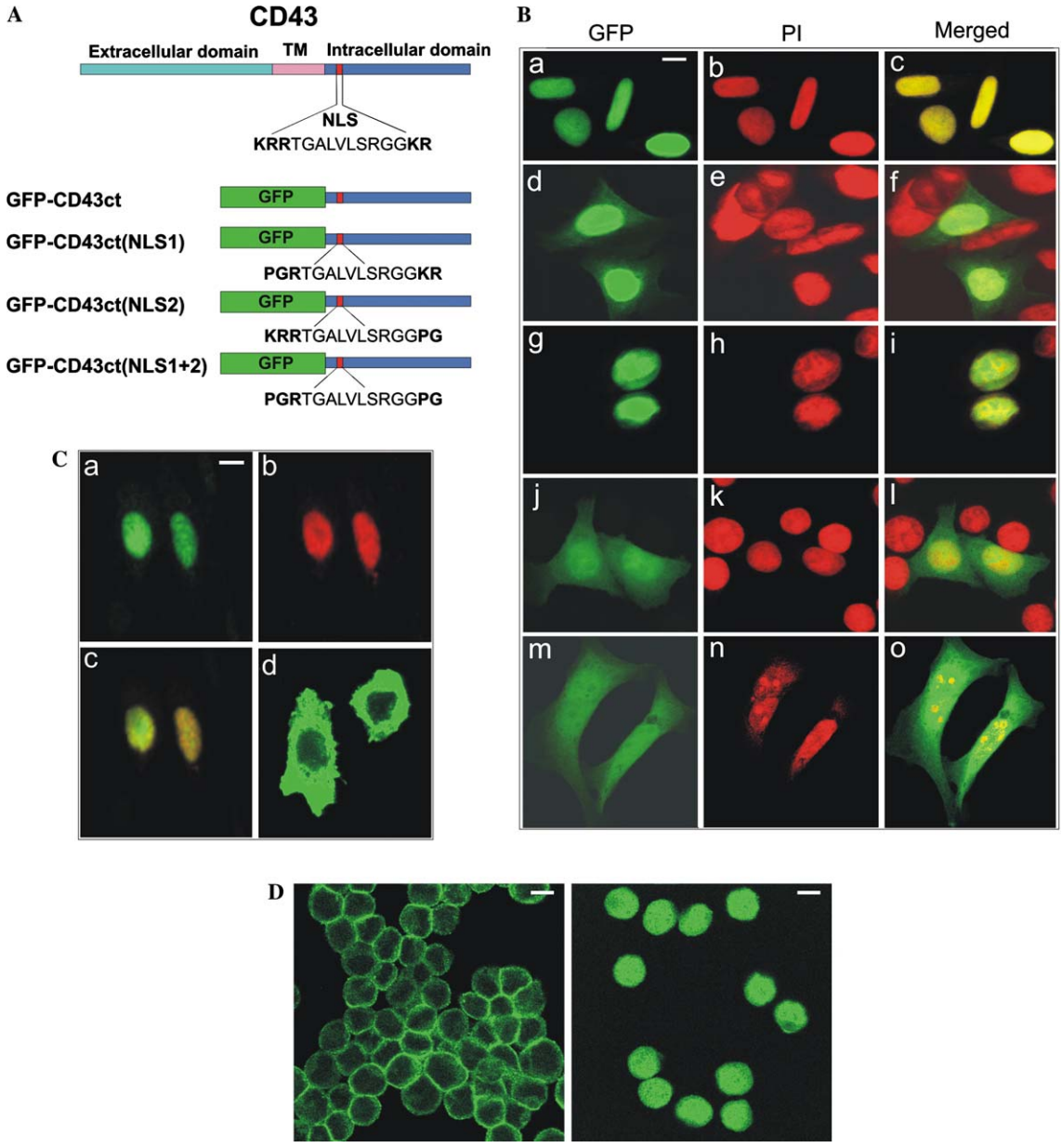

Fig. 1. Nuclear localization signal in the cytoplasmic tail of CD43 and its translocation to the nucleus. (A) Schematic figure showing the localization of the potential NLS in the CD43 sequence and the GFP-CD43ct expressed plasmids with mutations in the NLS sequence. (B) Confocal microscopy images of SW480 cells expressing the GFP-CD43ct constructs; GFP-CD43ct (a–c), GFP-CD43ct(NLS1) (d–f), GFP-CD43ct(NLS2) (g–i), GFP-CD43ct(NLS1+2) (j–l), and GFP alone (m–o). The PI panel shows the cell nucleus stained with propidium iodide (PI), giving red staining and the merged pictures are shown to the right. (C) Confocal microscopy images of CHO cells expressing GFP-CD43ct (a–c) and GFP alone (d). The nuclei in cells expressing GFP-CD43ct are stained with PI. (D) Confocal microscopy of COLO 205 cells stained with the αCD43-3A1 mAb at high density (left) and low density (right).

nucleus of SW480 cells was also found to bind to β-catenin (not with GFP alone), as immunoprecipitation using αGFP antibody could co-precipitate β-catenin (Fig. 2D). This further suggested that the cytoplasmic tail of CD43 can interact with β-catenin.

Transcriptional effect of CD43

To study if the interaction between CD43 and β-catenin had any functional effect on the transactivation properties of the β-catenin/TCF-4 complex in the nucleus, a luciferase reporter system was used. The pTOPFLASH vector has three optimal TCF-binding motifs upstream of a minimal c-Fos promoter driving luciferase expression [10]. By transfecting SW480 cells, expressing GFP-CD43ct or GFP alone, with pTOPFLASH an increased luciferase activity was found in cells expressing CD43ct. The negative control pFOPFLASH showed low activity (Fig. 3A). SW480-GFP cells had a slightly higher promoter activity than

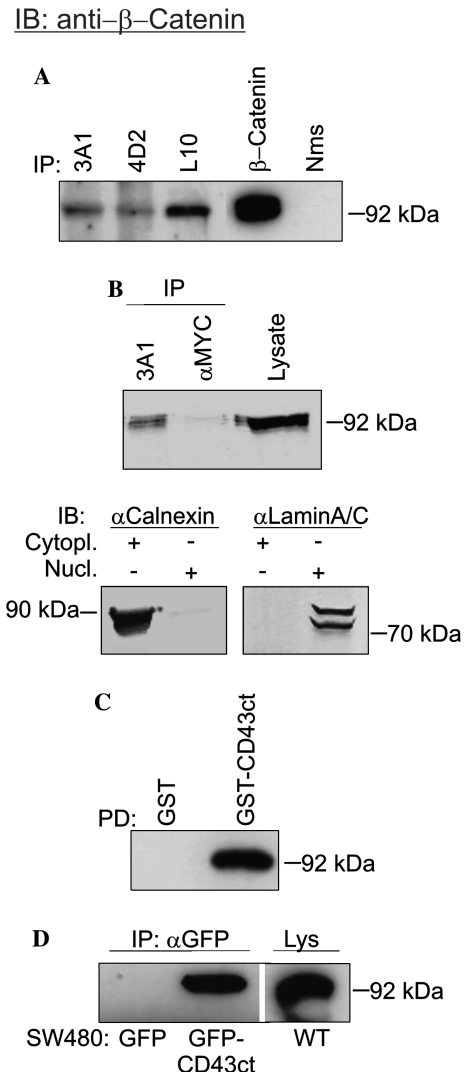

Fig. 2. CD43ct interacts with β-catenin. (A) Immunoprecipitations (IP) were made in lysates from COLO 205 cells with the mAb αCD43-3A1, αCD43-4D2, and αCD43-L10, followed by a Western blot probed with β-catenin antibody. Immunoprecipitations made with the β-catenin antibody and normal mouse serum (Nms) were used as positive and negative controls. (B) β-Catenin co-precipitation with CD43 in nuclear lysate from COLO 205 cells is shown by immunoprecipitation with the mAb αCD43-3A1 followed by Western blot with the β-catenin antibody. Negative immunoprecipitation control was αMyc mAb and lysate alone shows the β-catenin migration. Purity of the nuclear and cytoplasmic fractions was controlled by a Western blot using Calnexin and Lamin A/C antibodies. (C) Pull-down (PD) experiment using Sepharose-conjugated GST-fusion proteins (GST and GST-CD43ct) incubated in lysates from COLO 205 cells and analyzed by Western blot using the β-catenin antibody. (D) Lysates from SW480 cells expressing GFP and GFP-CD43ct were immunoprecipitated with αGFP antibody and analyzed by the β-catenin antibody by Western blot.

parental SW480 cells (not shown), indicating an effect of GFP alone. However, the higher luciferase signal in GFP-CD43ct cells compared to GFP cells together with the lower expression level (measured by GFP fluorescence, Fig. 3B) suggests a specific effect of CD43ct.

To further explore if the observed transcriptional upregulation of the β-catenin/TCF-4 promoter binding activity had any biological effect, the levels of the c-MYC and CyclinD1 protein expression were analyzed by flow cytometry. These two proteins are known to be induced via β-catenin/TCF-4 complex mediated activation. Figs. 3C and D show that SW480 cells expressing GFP-CD43 had a higher expression of both the c-MYC and CyclinD1 proteins compared to cells expressing GFP alone. CD43ct can thus not only bind β-catenin, but also affect TCF-4 transcriptional activity and increase the levels of the oncogenic proteins c-MYC and CyclinD1. Together, these results suggest that the nuclear localization of CD43ct enables it to modulate TCF-dependent gene expression by interaction with β-catenin.

Discussion

Here we report that the cytoplasmic domain of CD43 contains a functional bipartite NLS that can translocate it into the nucleus. Replacement of the basic amino acids in both clusters of the NLS in CD43ct inhibited the nuclear localization. That CD43ct could localize to the nucleus was further supported by the transcriptional activity affecting genes regulated by β-catenin/TCF-4 responsive promoters.

The intracellular domain of CD43 could interact with β-catenin, both at the cell surface and in the nucleus. Several proteins have been shown to interact with β-catenin and some of these affect the β-catenin/TCF-4 complex in the nucleus, either by promoting or suppressing gene expression. Two important target genes known to be activated by the β-catenin/TCF-4 complex are the proto-oncogenes *c-MYC* and *CyclinD1* [12,13]. That CD43ct could upregulate the promoter activity regulated by β-catenin/TCF-4 was observed with a luciferase reporter system. The protein levels of c-MYC and CyclinD1 were also increased in the presence of CD43ct. A role for CD43 and its nuclear localization in cell proliferation processes was also suggested by the variable cell localization due to the degree of confluence of the COLO205 cells. These results suggest that the nuclear localization of a CD43ct can have a true biological effect and that CD43 might have an oncogenic activity in colon cells.

An interesting protein in relation to CD43 is HIPK2 or STANK, a nuclear Ser/Thr kinase previously shown to interact with CD43 [14]. HIPK2 has been shown to stabilize the p53 protein and to increase the transcriptional activity of p53 target genes [15,16] and recently CD43 expression was also found to induce the activation of p53 [17]. The HIPK transcription regulators are closely related to each other and the HIPK1 is important for regulating the activity of the transcription factor

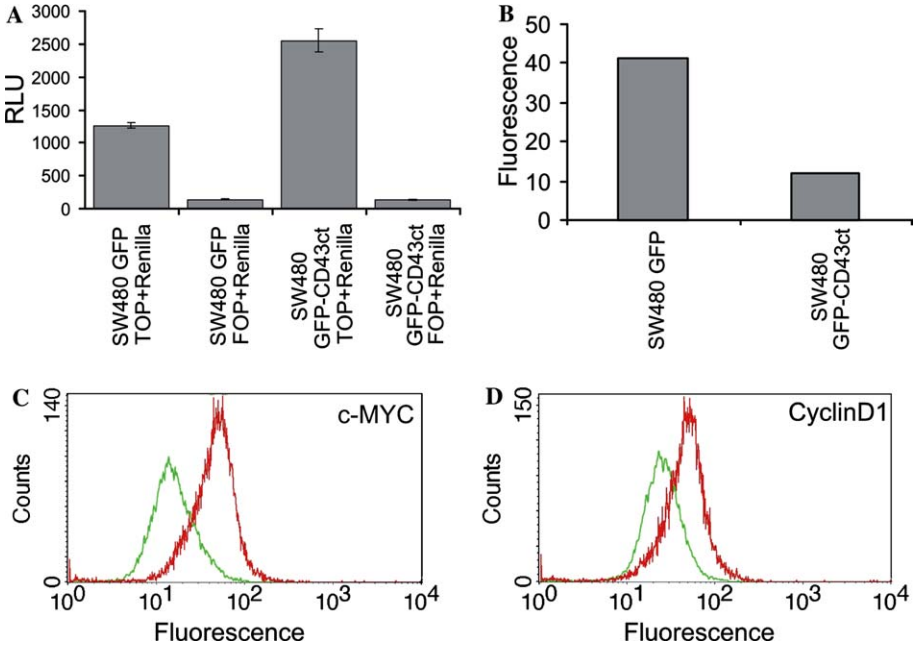

Fig. 3. β-Catenin/TCF-4 targeted genes are affected by the presence of CD43ct. (A) The luciferase reporter plasmids pTOPFLASH and pFOP-FLASH were transfected into SW480 cells expressing GFP or GFP-CD43ct and the luciferase activity was measured. (B) The GFP fluorescence in the SW480 cells expressing either GFP or GFP-CD43ct as used in (A) was measured by FACS and used to compare the relative expression levels. (C,D) The SW480 cells expressing either GFP or GFP-CD43ct were analyzed by FACS for the protein levels of c-MYC and CyclinD1. The gray (green) line represents SW480 GFP and black (red) line SW480 GFP-CD43ct expressing cells.

DAXX [18]. The DAXX protein has also been shown to interact with the cytoplasmic domain of CD43 and thereby inhibit CD43-mediated apoptosis [19]. Until now, it has not been possible to understand how the cell membrane protein CD43 could interact with the nuclear proteins HIPK2 and DAXX. The present observation of a functional NLS of CD43ct cast new light on how this might function.

How can CD43, a transmembrane protein, appear in the nucleus? For several type I transmembrane proteins, e.g., Notch-1, amyloid precursor protein, ErbB-4 and CD44, a processing pathway has been described which entails proteolytic cleavage in the membrane, release and nuclear translocation of the intracellular domain, ultimately resulting in changes in gene transcription. This novel signaling pathway is called regulated intramembrane proteolysis (RIP). The process usually starts with a proteolytic release of the ectodomain, e.g., by metalloproteases, followed by an intramembrane cleavage by γ-secretase. As it has previously been shown that CD43 can be cleaved extracellularly at the cell surface in hematopoietic and colon cancer cells [20–22] it is not unlikely that a similar series of events also occur for CD43. We therefore propose a mechanism for CD43 signaling involving membrane-associated cleavages and a translocation of CD43ct to the nucleus as outlined in Fig. 4.

The absence of CD43 expression in normal colon and appearance in colon adenoma cells [7] suggest that

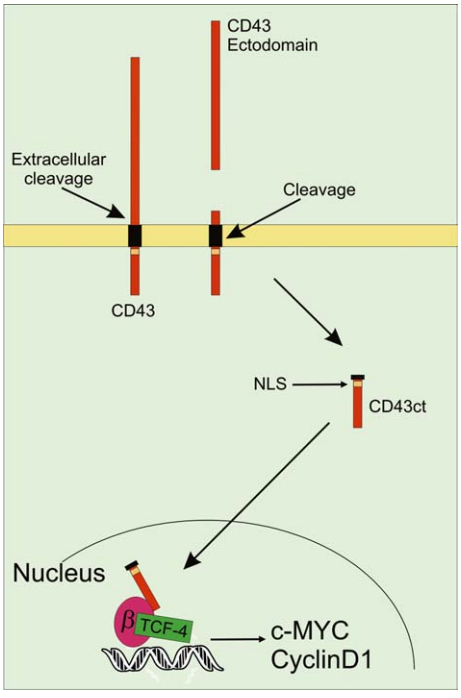

Fig. 4. Hypothesis for CD43 nuclear signaling. CD43 may undergo proteolytic cleavages, releasing the CD43ct from the membrane. CD43ct contains a NLS sequence directing it into the nucleus, where it interacts with β-catenin and causes an altered gene expression.

CD43 could be involved in early stages of adenoma formation. This is concordant with the localization of CD43 in colon cancer cell lines, where sometimes, like in

COLO 205 cell, only nuclear staining is observed. However, this localization is only found in cells at low confluency. Leukocyte-type cell lines and leukocytes in tissues show membrane staining irrespective of proliferative status. This suggests that the observed nuclear localization and mechanisms behind this depend on cell type and appear to be specific for at least colon adenoma and carcinoma cells. This and the binding of β-catenin to CD43 and its transcriptional effects inducing the promitotic proteins c-MYC and CyclinD1 suggest that CD43 could have a role in colon adenoma formation.

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