Journal of Cellular Biochemistry 103:957–971 (2008)

Compartmentalization in Membrane Rafts Defines a Pool of N-Cadherin Associated With Catenins and Not Engaged in Cell–Cell Junctions in Melanoma Cells

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Abstract Melanoma progression is associated with changes in adhesion receptor expression, in particular upregulation of N-cadherin which promotes melanoma cell survival and invasion. Plasma membrane lipid rafts contribute to the compartmentalization of signaling complexes thereby regulating their function, but how they may affect the properties of adhesion molecules remains elusive. In this study, we addressed the question whether lipid rafts in melanoma cells may contribute to the compartmentalization of N-cadherin. We show that a fraction of N-cadherin in a complex with catenins is associated with cholesterol/sphingolipid-rich membrane microdomains in aggressive melanoma cells in vitro and experimental melanomas in vivo. Partitioning of N-cadherin in membrane rafts is not modulated by growth factors and signaling pathways relevant to melanoma progression, is not necessary for cell–cell junctions' establishment or maintenance, and is not affected by cell–cell junctions' and actin cytoskeleton disruption. These results reveal that two independent pools of N-cadherin exist on melanoma cell surface: one pool is independent of lipid rafts and is engaged in cell–cell junctions, while a second pool is localized in membrane rafts and does not participate in cell–cell adhesions. Targeting to membrane rafts may represent a previously unrecognized mechanism regulating N-cadherin function in melanoma cells. J. Cell. Biochem. 103: 957–971, 2008.

Key words: melanoma; N-cadherin; membrane microdomains

Cutaneous melanoma is an aggressive tumor with a high propensity to invade and metastasize. Melanoma progression commonly proceeds through stages that are well described morphologically, but still poorly characterized in molecular and biochemical terms [Hsu et al., 2002]. The transformation of radial growth phase (RGP) melanomas, non-invasive tumors confined to the epidermis, into vertical growth phase (VGP) melanomas coincides with the appearance of invasive properties and tumor spreading into the dermis and subsequently to distant organs. At the molecular level melanoma progression is consistently associated with de novo expression of αvβ3 integrin [Albelda et al., 1990], E-cadherin to N-cadherin switch [Hsu et al., 1996], and acquired responsiveness to several tumor- and stromal-derived growth factors (GFs) [Li et al., 2003; Stove et al., 2003]. Upregulation of N-cadherin appears to promote melanoma progression through several mechanisms. In fact, N-cadherin allows melanoma cells to interact with N-cadherin-expressing dermal fibroblasts resulting in facilitated migration into the dermis [Li et al., 2001], and it favors melanoma cell survival through the activation of Akt [Li et al., 2001].

Lipids are distributed within the plasma membrane according to their biophysical properties.

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

Grant sponsor: Swiss National Science Foundation; Grant number: 3100A0-101739; Grant sponsor: MEDIC Foundation.

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Received 4 June 2007; Accepted 6 June 2007

DOI 10.1002/jcb.21463

In particular, cholesterol and sphingolipids can tightly assemble in discrete regions of the plasma membrane called membrane microdomains, or lipid rafts [Brown and London, 2000]. Since only certain proteins have affinity for membrane rafts, these regions can function in organizing signaling complexes [Brown and London, 2000] [Rajendran and Simons, 2005]. The involvement of lipid rafts in signaling regulation has in fact been established for several receptors in various cell types (e.g., T cell receptor in T lymphocytes, IgE in mast cells and basophils, Glial-cell-derived neurotrophic-factor receptors in neurons, etc.) [Simons and Toomre, 2000]. Two recent studies have shown that N-cadherin is in part associated with cholesterol/sphingolipid rich membrane rafts in cerebellar granule cells, dorsal root ganglion neurons, and primary myoblasts. In one study membrane raft disruption in the neural growth cone prevented N-cadherin-dependent growth cone motility [Nakai and Kamiguchi, 2002]. In the second study N-cadherin association with membrane rafts was shown to promote cell–cell junction assembly between myoblasts [Causeret et al., 2005]. These works strongly indicate that N-cadherin function can be regulated in membrane rafts, and that the biological consequences of this regulation are further dictated by the cellular context.

This study was initiated to examine the putative role of membrane rafts in regulating N-cadherin compartmentalization in melanoma cell plasma membrane. We hypothesized that during tumor development, including melanoma, the compartmentalization in membrane rafts of proteins involved in tumor progression could represent an additional mechanism regulating their function, for example, by modulating their interactions with other transmembrane proteins or regulating assembly of signaling complexes, and thereby affecting cell behavior. We show here that a fraction of N-cadherin in a complex with catenins is localized in membrane microdomains of some melanoma cells in vitro and experimental melanomas in vivo. We also show that the interaction with raft lipids is not necessary for, and does not require N-cadherin engagement in cell–cell junctions. Our results suggest that recruitment of N-cadherin to membrane microdomains of melanoma cells may regulate its function outside cell–cell junctions, thus conferring N-cadherin so far undescribed properties possibly affecting melanoma cell biology and promoting malignant progression.

MATERIALS AND METHODS

Reagents and Antibodies

Methyl-β-cyclodextrin (MβC), rec human insulin-like growth factor-I (IGF-I), LY-294-002, cytochalasin D, filipin, fibronectin were from Sigma-Aldrich, H-89, PP2, goat anti-IGFR were from Calbiochem, forskolin and PD-98059 from Biomol, rec human basic fibroblast growth factor (bFGF) from Peprotech, rec human heregulin1 (HRG1) from R&D systems; the antibodies used in this work were: mouse anti- N-cadherin, E-cadherin, β-catenin, p120-catenin, α-catenin, phosphotyrosine (RC20), rabbit anti-cav-1, all purchased from BD Biosciences; rabbit anti-N-cadherin and anti-panSrc were obtained from Santa Cruz Biotech, mouse anti-TrR from Zymed, anti-ERKs/phosphoERKs from Cell Signaling, biotinylated rat anti CD-31 from Pharmingen.

Cell Culture

Human cell lines were used in this study. RGP melanoma cell line SBCl2 (a kind gift of Dr. B. Giovanella, Sthelin Foundation, Houston), VGP cell lines Me300 and Me191 (kindly provided by Dr. D. Rimoldi, Ludwig Institute, Epalinges, Switzerland), and metastatic melanoma cells SK-Mel-28 (ATCC) were grown in RPMI supplemented with 10% fetal calf serum (FCS). The melanoma cell lines WM35 (RGP), WM793 (RGP/VGP), WM115 (VGP), WM239A (metastasis; kindly provided by Dr. M. Herlyn, Wistar Institute, Philadelphia) were grown in Tu 2% as described [Satyamoorthy et al., 2003]. Cells were serum-starved for 16 h before treatment with GFs or PD-98059.

DRMs Preparation

Cells were lysed in TX-MN buffer (25 mM MES pH 6.5, 150 mM NaCl, 1% Triton X-100) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) on ice and homogenized in a Dounce homogenizer. Lysates (1.6–2 mg proteins in 1 ml) were adjusted to 45% sucrose in MN, and a step gradient of 5–30% sucrose was layered above it. After centrifugation at 200,000*g* (Sorvall rotor TST 55.5, 16 h, 4°C), 12 fractions were collected from the top of the gradients. To disrupt membrane rafts SK-Mel-28 and WM793 cells were treated with 20 or

10 mM MβC respectively for 1 h. Aliquots of each fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot (WB), or subjected to immunoprecipitation as described below. Tumors were briefly washed in cold PBS, and then homogenized in TX-MN buffer. Lysates were cleared at 4,500 rpm for 5 min before sucrose gradient centrifugation as above.

Cell Extracts, Immunoprecipitation, Immunoblotting, and Cell Surface Biotinylation

For detection of proteins in total cell lysates, cells were lysed in modified RIPA buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycolate, (DOC) and a protease inhibitor cocktail). For immunoprecipitation (IP), after lysis in Triton-DOC buffer (above buffer without SDS), cell extracts were precleared with Protein A-Sepharose, and then incubated in the presence of primary antibodies and Protein G-Sepharose (Amersham), for 3 h at 4°C. Cell surface biotinylation was performed by incubating adherent cells with EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce, 0.3 mg/ml in serum/glutamine-free medium) for 20 min on ice. Cells were then washed in Tris HCl 25 mM to quench biotin, and processed for sucrose gradient centrifugation. For immunoprecipitation after sucrose gradient centrifugation, fractions 2–5 and Triton-soluble fractions 9–12 (obtained from 6 mg of total proteins) were pooled and diluted (1:1 and 1:2, respectively) in 1% Triton X-100 in 20 mM Tris-HCl pH 8, before addition of antibodies and Protein G-Sepharose, or of Streptavidin-Sepharose for isolation of biotinylated proteins. Immunoprecipitations were carried out for 4 h at 4°C. Immunoprecipitates and total lysates were analyzed by SDS–PAGE and WB with appropriate antibodies, that were visualized after incubation with the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Microscopy

To analyze the effect of raft disruption on cell–cell junctions stability, cells plated on coverslips were treated with MβC (10 mM for SBCl2 and WM793, 20 mM for SK-Mel-28) for 1 h, fixed in 2% p-formaldehyde, permeabilized in 0.3% Triton X-100, and incubated with primary antibodies followed by anti-mouse Alexa488 or anti-rabbit Alexa546 secondary antibodies (Molecular Probes). Cholesterol was detected by incubation with 0.05 mg/ml filipin in PBS. To analyze cell–cell junctions reassembly after raft and junctions disruption, cells were treated with MβC for 70 min and 2.5 mM EDTA for the last 5–10 min in serum-free medium; control cells were treated with 2.5 mM EDTA only. Cells were then collected and plated on fibronectin (5 μg/ml)-coated coverslips in serum-free medium containing 0.5 mM $CaCl_2$ for 75 min before fixing and staining. Coverslips were mounted in ProLong Antifade (Molecular Probes) and analyzed using a 63× or 100× oil-immersion objective on a fluorescent microscope (Zeiss Axioplan) equipped with AxioCam MRm (Zeiss). Images were assembled using Adobe Photoshop version 7.0.

In Vivo Tumor Formation and Histological Analysis

SK-Mel-28 cells (10^6) in serum-free medium were injected sub-cutaneously (s.c.) in the flank of Swiss-nu/nu mice (Charles River) ($n = 4$). Tumors were harvested after 2–3 weeks growth at volumes of approximately 0.2 cm^3. Two tumors were separately processed for DRMs isolation, the other two were either fixed in 3% PFA and embedded in paraffin, or embedded in OCT and frozen in dry ice for immunohistochemistry with anti-CD31 antibody and standard HE staining. Animal experiments were performed in accordance with local regulation.

RESULTS

A Fraction of N-Cadherin Is Associated With Membrane Microdomains of Melanoma Cells

We first analyzed the expression levels of N-cadherin in several melanoma cell lines derived from tumors at different stages of progression. It was shown that melanoma cells maintain in vitro many of the biochemical and biological properties of the tumors they were derived from Satyamoorthy et al. [1997]: in fact, contrary to VGP and metastatic melanoma cells, RGP melanoma cells usually do not grow in soft agar, are poorly or not tumorigenic in nude mice, and express only low levels of αvβ3 integrin and N-cadherin. All cell lines examined express N-cadherin, including the two RGP melanoma cell lines SBCl2 and WM35, while E-cadherin was detectable in WM35 cells only. All cells also express Transferrin receptor (TrR) and caveolin-1 (cav-1), known to partition in plasma

membrane regions excluding or consisting of cholesterol/sphingolipid-rich domains, respectively [Smart et al., 1995] (Fig. 1a). We then analyzed the distribution of N-cadherin, TrR, and cav-1 in membrane microdomains isolated as detergent resistant membranes (DRMs) on the basis of their insolubility in non-ionic detergents [London and Brown, 2000]. Triton X-100 lysates of SBCl2 and SK-Mel-28 cells were subjected to sucrose gradient centrifugation, and DRMs corresponding to cholesterol/sphingolipid-rich membrane microdomains were identified in low-density fractions 2–5 that contain cav-1 but not TrR. We found that most of N-cadherin is present in Triton-soluble fractions (fractions 7–12) in both RGP melanoma cells SBCl2 and metastatic melanoma cells SK-Mel-28. N-cadherin is also present in DRMs (fractions 2–5) of SK-Mel-28 cells, but not SBCl2 cells, despite similar expression levels in the two cell lines (Fig. 1b). N-cadherin association with DRMs was lost upon disruption of membrane rafts with the cholesterol-sequestering agent MβC, indicating that co-fractionation of N-cadherin with DRMs is dependent on its interaction with cholesterol/sphingolipid-rich regions (Fig. 1c). Cholesterol extraction did not significantly increase Triton X-100 solubility of cav-1. This was already observed [Scheiffele et al., 1997] and could be due to the tendency of cav-1 to oligomerize, as well as to its possible engagement in complex protein–protein interactions that make it highly detergent-insoluble in the conditions used. The integrin αvβ3, another marker of melanoma progression, was not detected in DRMs of either cell line (data not shown), suggesting that N-cadherin partitioning to melanoma lipid rafts is not a random event but rather a specific process. Since cholesterol/sphingolipid-rich microdomains are also present on the membrane of some intracellular compartments, such as caveosomes, endosomes, and the Golgi, we examined if lipid-rafts associated N-cadherin was indeed localized at the plasma membrane. To this purpose we labeled cell surface proteins with biotin in conditions in which endocytosis was prevented (i.e., by incubating the cells on ice), and then extracted DRMs. Biotinylated proteins from both DRMs and Triton X-100 soluble fractions were captured by streptavidin columns and probed for N-cadherin and TrR. This experiment revealed that a fraction of N-cadherin associated with DRMs was biotinylated, indicating that lipid-rafts associated N-cadherin is indeed present at the cell surface (Fig. 1d). We cannot exclude, however, that N-cadherin is also partly associated with intracellular lipid rafts, for example, during its transport to the cell surface or during endocytosis. Biotinylated TrR was found only in Triton X-100-soluble fractions, thus confirming the proper isolation of DRMs. To collect evidence that the association of N-cadherin with lipid rafts is not unique to SK-Mel-28 cells, we examined the presence of N-cadherin in membrane rafts of other melanoma cell lines. For all cell lines DRMs were isolated from Triton X-100 lysates containing 2 mg of proteins, and the same volumes of DRM- and Triton X-100-soluble fractions ("standard volumes") were analyzed in each experiment by SDS–PAGE. We found that a fraction of N-cadherin is localized in DRMs in all additional cell lines tested with the exception of WM35 cells, which express low levels of this protein (Fig. 2a). N-cadherin remained undetectable in DRMs of WM35 cells also when we analyzed volumes of DRM fractions two times larger than the "standard" volumes, while it was detectable in "standard" volumes of WM239A DRM fractions loaded on the same gel (Fig. 2b). WM239A cells were chosen for this direct comparison because among the cell lines tested they express levels of N-cadherin closer to those found in WM35 cells, and their levels of cav-1 are similar to those found in WM35 cells. E-cadherin was not detected in DRMs of either WM35 cells, where it is mostly cytoplasmic and not engaged in cell–cell junctions [Smalley et al., 2005], or HaCat keratinocytes (Fig. 2c). These results show that N-cadherin is localized to membrane microdomains of six out of eight melanoma cell lines here examined. Importantly, the interaction with membrane rafts is not related to N-cadherin expression levels.

In order to exclude that our observations were dependent on the in vitro culture system, we examined N-cadherin association with membrane rafts of experimental melanomas in vivo. For this purpose, we injected SK-Mel-28 cells s.c. in nude mice, and after 2 weeks we excised the tumors and isolated DRMs. As shown in Figure 3a, N-cadherin was detected in DRMs, indicating that its association with membrane microdomains occurs in vivo and it is not an artifact of the in vitro culture system. To estimate the contribution of tumor stroma

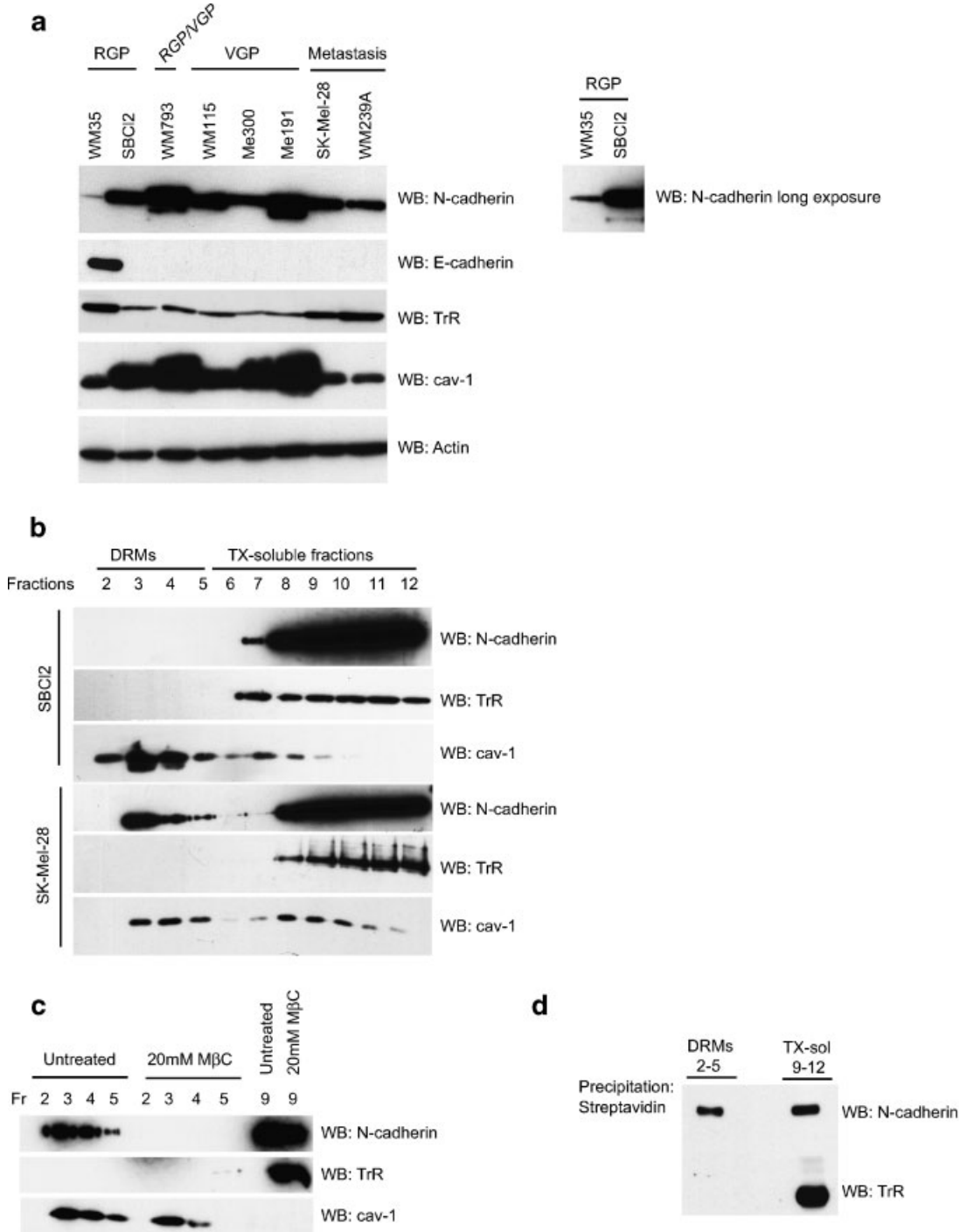

Fig. 1. N-cadherin is associated with cholesterol/sphingolipid-rich membrane microdomains in SK-Mel-28 melanoma cells. **a**: Expression levels of N-cadherin, E-cadherin, TrR and cav-1 in melanoma cell lines. A longer exposure better evidentiates N-cadherin expression in WM35 cells. The corresponding tumor stage is indicated for each cell line. **b**: Analysis of N-cadherin partitioning in Triton X-100-soluble and insoluble fractions derived from melanoma cell lysates (2 mg of total proteins). DRMs representing membrane rafts were identified by the presence of cav-1 and the absence of TrR. **c**: Disruption of membrane rafts in SK-Mel-28 cells by MβC treatment (1 h) prevented co-fractionation of N-cadherin with low density, Triton X-100-insoluble material. **d**: Isolation of biotinylated cell surface proteins showed that N-cadherin is associated with plasma membrane lipid rafts. Biotinylated TrR was recovered only from Triton X-100-soluble fractions.

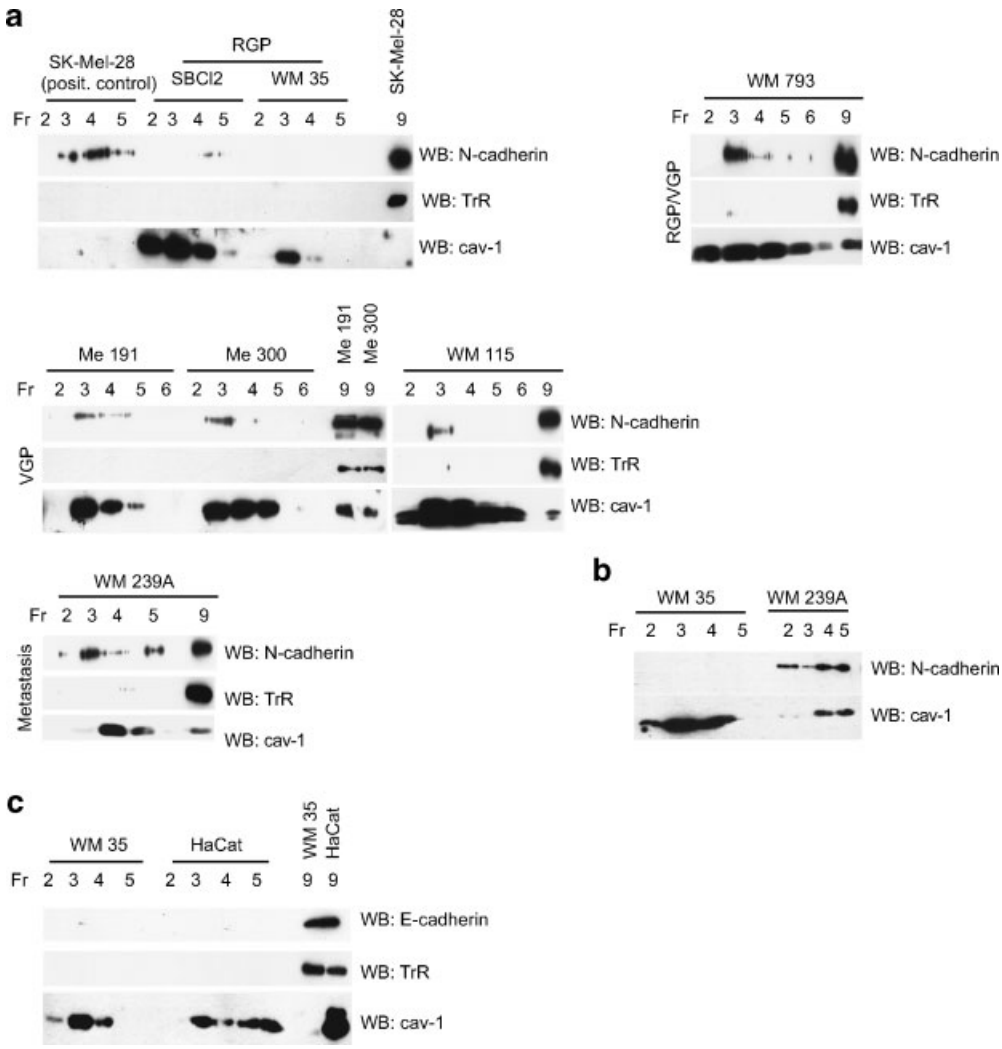

Fig. 2. **a**: Analysis of N-cadherin partitioning in DRMs of melanoma cell lines. DRMs-containing fractions from metastatic SK-Mel-28 cells were loaded next to DRMs derived from RGP cell lines as a positive control. The stage of the tumor of origin is indicated for each cell line. DRMs were isolated from lysates containing 2 mg of proteins for all cell lines. In (**b**) the volumes of DRM fractions from WM35 cells that were loaded on the gel were 2× larger than those of the corresponding fractions from WM239A cells. **c**: E-cadherin is not detected in DRMs of WM35 melanoma cells and of HaCat keratinocytes. The absence of TrR in DRM preparations in all experiments was tested as a quality control.

and tumor-associated vessels (both known to express N-cadherin) to the amount of N-cadherin detected in the whole xenografts, we stained SK-Mel-28-derived tumor sections with hematoxylin–eosin (HE) and with an antibody specific for the endothelial cell marker CD31. HE staining showed that tumors consisted largely of melanoma cells with only minimal presence of stroma, and staining with an antibody for the endothelial cell marker CD31 revealed limited tumor vascularization at this stage of tumor growth (Fig. 3b). These observations support the conclusion that the fraction of N-cadherin detected in DRMs isolated from the whole xenografts was mostly tumor-associated, with only a small, if any, possible contribution from mouse stromal tissue and vessels.

N-Cadherin Association With DRMs Is Not Modulated by Signaling Pathways Implicated in Melanoma Development

We reasoned that the activation of GF-dependent signaling during melanoma cell transformation might influence the distribution of N-cadherin at the cell membrane. Several GFs have been shown to promote and sustain melanoma cell malignancy [Li et al., 2003; Stove

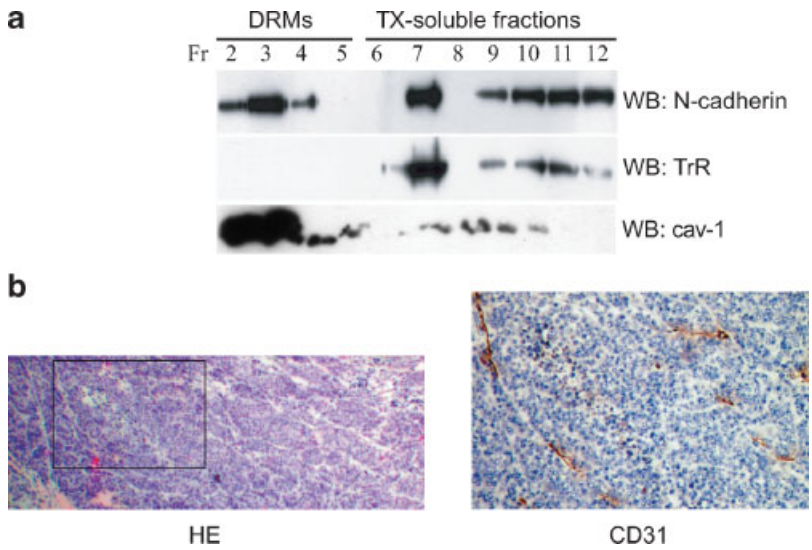

Fig. 3. **a**: N-cadherin association with DRMs isolated from SK-Mel-28 derived tumors. **b**: HE and anti-CD31 staining (enlargement of framed area in HE picture) of SK-Mel-28-derived tumors developed in parallel with those subjected to rafts extraction revealed limited stromal infiltration and tumor vascularization.

et al., 2003], and their receptors have often been detected in membrane rafts [Pike, 2005]. We found that SK-Mel-28 cells express the receptors FGFR-1, IGFR, and ErbB2, but not EGFR, and Met (data not shown). These receptors are functional, as shown by IGFR and ErbB2 increased tyrosine phosphorylation in response to IGF-I and HRG1 respectively, and by Akt activation in response to bFGF (Fig. 4a and data not shown). In order to examine if activation of these GFRs can affect the association of N-cadherin with membrane rafts, we treated serum-starved SK-Mel-28 cells with bFGF, IGF-I, HRG1, or FCS for different times (30 min, 90 min, 3 h, 6 h) and then isolated DRMs. The amount of N-cadherin in DRMs was not changed by stimulation with any of these GFs (Fig. 4a and data not shown). Since the MAPK pathway is often constitutively active in melanoma cells due to mutations in N-Ras [Ball et al., 1994] or B-Raf, or to constitutive GFR activation [Satyamoorthy et al., 2003], we examined if inhibition of this pathway affects N-cadherin partitioning in DRMs. ERKs are in fact constitutively phosphorylated in both SK-Mel-28 and WM793 cells in the absence of serum, and treatment of these cells with the MEK inhibitor PD-98059 partially reduced ERK phosphorylation. We observed that treatment with PD-98059 decreased ERK phosphorylation but had no effect on N-cadherin association with DRMs in both SK-Mel-28 and WM793 cells at 30 min (Fig. 4b). Inhibition of ERKs for shorter (5 min) or longer (1, 5, 22 h) periods also did not affect N-cadherin association to DRMs (supplementary Fig. 1). In order to examine if other signaling pathways known to contribute to cell transformation may affect N-cadherin localization to membrane rafts, we treated SK-Mel-28 cells with pharmacological agents interfering with protein kinase A (PKA), Src family kinases (SFKs), and phosphatidylinositol 3-kinase (PI 3-K) activities (Table I). None of the drugs tested affected the amount of N-cadherin co-fractionating with DRMs (data not shown). The localization of cav-1 and TrR was monitored in all the experiments here described for quality control of our DRM preparations.

These results indicate that the association of N-cadherin with membrane rafts of SK-Mel-28

TABLE I. Chemicals and GFs Tested for Their Effect on N-Cadherin Association With Membrane Rafts

Chemical	Function	Concentration
H-89	PKA inhibitor	3 μM
Forskolin	PKA activator	40 μM
PP2	Src-family kinase inhibitor	5 μM
LY-294002	PI3-K inhibitor	10 μM
PD-98059	MEK inhibitor	10 μM
IGF-I		50 ng/ml
HRG1		20 ng/ml
bFGF		20 ng/ml

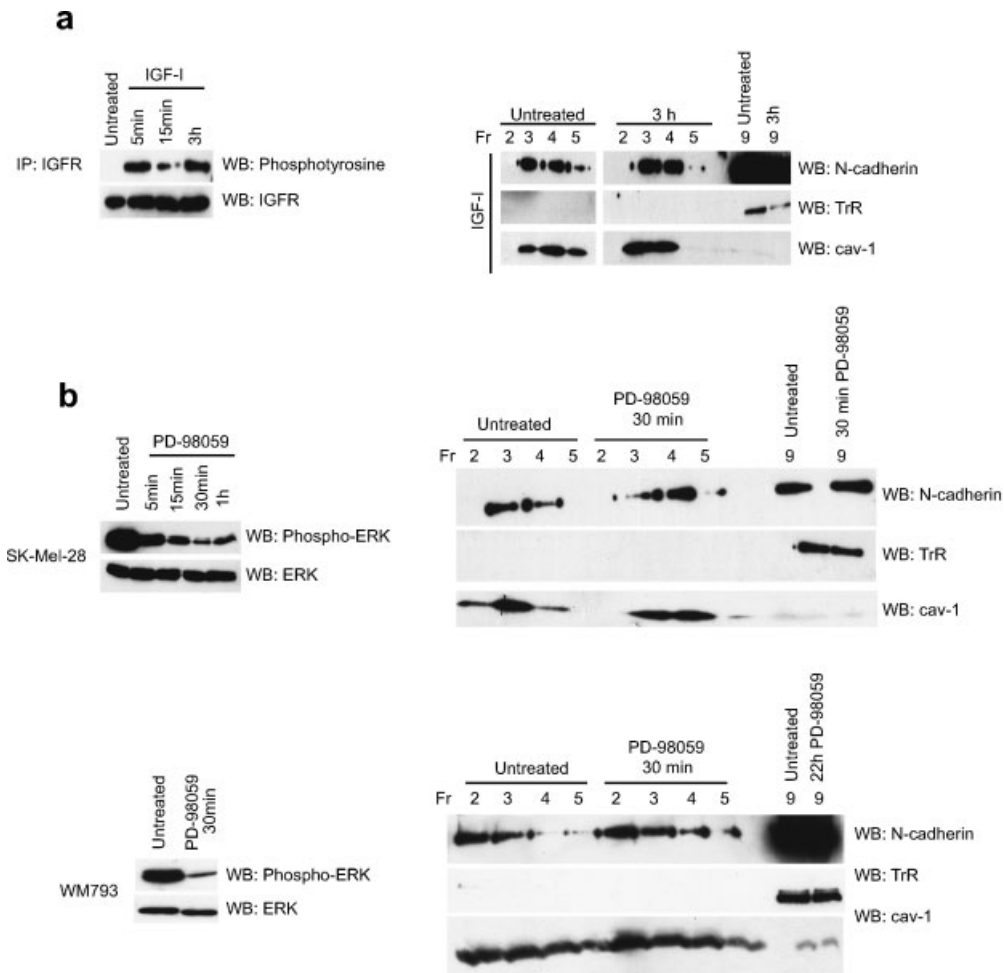

Fig. 4. The association of N-cadherin with membrane rafts is not affected by IGF-I treatment or by ERK inhibition. **a**: Increased tyrosine phosphorylation of IGFR immunoprecipitated from SK-Mel-28 cells after IGF-I treatment; analysis of N-cadherin partitioning in DRMs (from 1.8 mg of total proteins) of SK-Mel-28 cells in response to IGF-I. Samples from sucrose gradient centrifugation were loaded on distant portions of the same gel. **b**: Inhibition of ERKs upon treatment of SK-Mel-28 and WM793 cells with PD-98059 for different times was indicated by decreased ERK phosphorylation; ERK inhibition for 30 min did not affect the association of N-cadherin with DRMs in either cell line. The quality of DRM preparations (from 2 mg of total proteins for both cell lines) was evaluated by analyzing TrR and cav-1 distribution in the collected fractions.

cells is not dependent on signaling downstream of FGFR1, IGFR, or ErbB2, or involving PKA, PI3-K, or SFKs, thus excluding a significant regulatory role for main GFs and signaling pathways implicated in melanoma malignancy. Moreover, ERK-dependent signaling, which plays a major role in melanoma development, does not influence the association of N-cadherin with membrane rafts in two distinct melanoma cell lines, SK-Mel-28 and WM793.

The Fraction of N-Cadherin Associated With Membrane Microdomains Interacts With Catenins

In order to obtain evidence for a possible biological role of N-cadherin present in membrane rafts, we examined whether lipid-rafts localized N-cadherin interacts with other proteins. We thus performed a series of immunoprecipitations examining at first if N-cadherin could be isolated from DRMs in a complex with catenins, the best characterized cadherins binding-partners. As shown in Figure 5a, N-cadherin was immunoprecipitated in a complex with β-catenin from DRMs derived from SK-Mel-28 and WM793 cells. SFKs (known to be present in rafts) could not be co-immunoprecipitated with N-cadherin, indicating that the association of N-cadherin to β-catenin in DRMs is specific. N-cadherin was also detected in β-catenin immunoprecipitates from DRMs of

SK-Mel-28 cells (Supplementary Fig. 2). Since we were unable to detect p120-catenin and α-catenin in N-cadherin immunoprecipitates from DRMs (data not shown), we immunoprecipitated p120-catenin and α-catenin from DRMs and Triton X-100-soluble fractions derived from SK-Mel-28 cells and blotted for N-cadherin. We detected N-cadherin in p120-catenin immunoprecipitates, and β-catenin in α-catenin immunoprecipitates, whereas N-cadherin could not be co-precipitated with α-catenin (Fig. 5b and data not shown). In all the experiments described proper isolation of DRMs was confirmed by the absence in these fractions of TrR

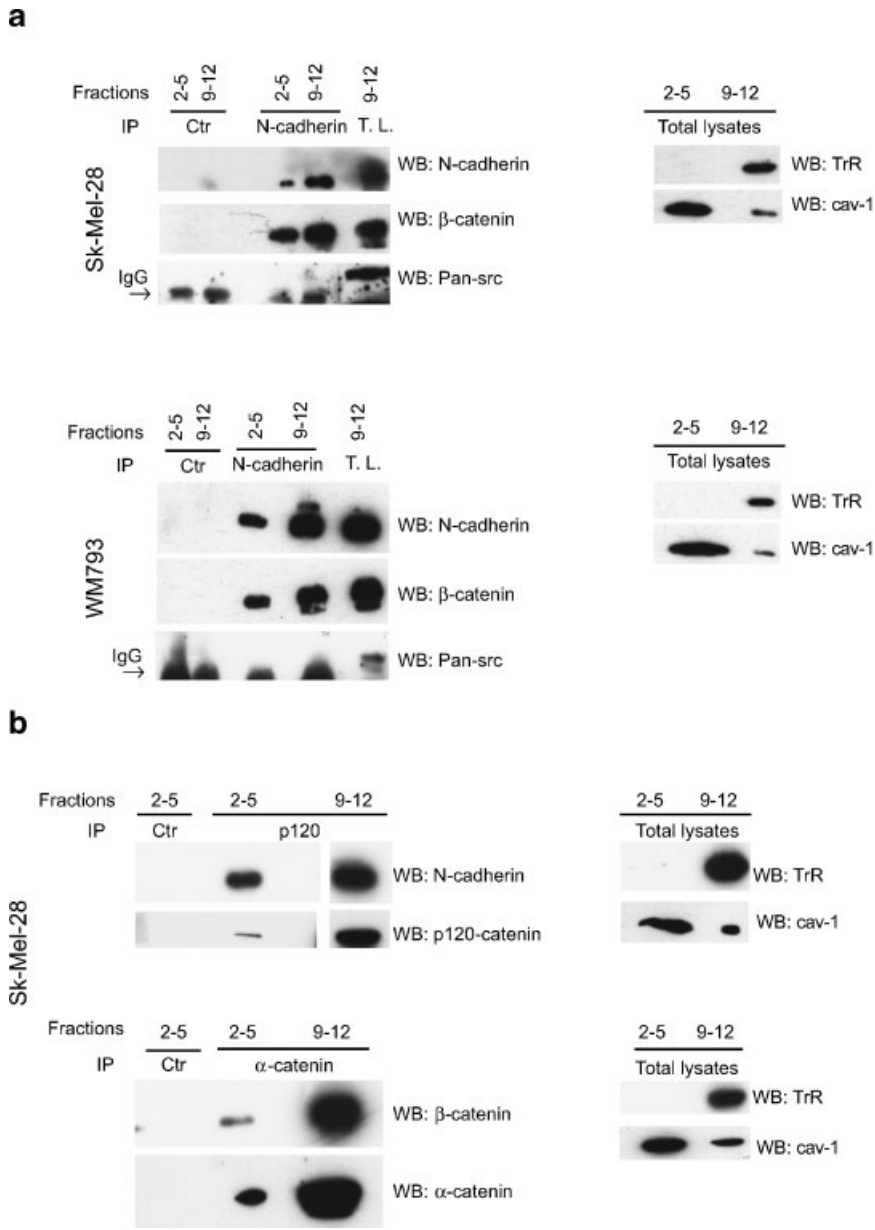

Fig. 5. Co-immunoprecipitation of N-cadherin with catenins from DRMs. **a**: After sucrose gradient centrifugation of SK-Mel-28 and WM793 lysates, fractions 2–5 containing DRMs and Triton X-100-soluble fractions 9–12 were subjected to immunoprecipitation with anti-N-cadherin antibodies; as a control (Ctr), DRMs, and Triton X-100-soluble fractions were immunoprecipitated with mouse IgG. WB revealed the association of N-cadherin with β-catenin in DRMs of both cell lines. SFKs did not co-immunoprecipitate with N-cadherin. **b**: DRMs and Triton X-100-soluble fractions from SK-Mel-28 cells were precipitated with anti-p120- or anti-α-catenin antibodies; WB analysis revealed the association of N-cadherin with p-120-catenin and of β-catenin with α-catenin in DRMs. The absence of TrR in DRMs samples (total lysates 2–5) was confirmed.

(and the presence of cav-1; Fig. 5 right panels). These results show that N-cadherin present in membrane rafts is associated with β-catenin and p120-catenin, and it might also indirectly interact with α-catenin through β-catenin.

The Association With Membrane Rafts Is Not Necessary for N-Cadherin Engagement in Cell–Cell Junctions

In myoblasts the association of N-cadherin with membrane rafts is dependent on the clustering of N-cadherin in Adherens Junctions, and it is also required for efficient Adherens Junctions establishment [Causeret et al., 2005]. We reasoned that in melanoma cells N-cadherin partitioning in membrane microdomains might have a different biological function, because also WM35 [Smalley et al., 2005] and SBCl2 cells assemble N-cadherin-containing cell–cell junctions, despite the fact that N-cadherin is not detectable in their DRMs. On the other hand, the isolation of a N-cadherin–catenin complex from membrane rafts of SK-Mel-28 cells prompted us to investigate if the fraction of N-cadherin localized to membrane rafts in melanoma cells is functionally related to the fraction engaged in cell–cell adhesions. To this purpose we performed two types of experiments: first, we tested if disruption of cell–cell junctions or of the actin cytoskeleton affected N-cadherin partitioning in DRMs; second, we examined if disruption of membrane rafts by MβC destabilized cell–cell adhesions or prevented their assembly. SK-Mel-28 cells were treated with the Ca^{2+} chelator EGTA until they dissociated from each other and rounded up, while still remaining attached to the culture dish. This treatment did not affect N-cadherin association with DRMs (Fig. 6a). To investigate if the fraction of N-cadherin in DRMs of EGTA-treated cells is at the cell membrane, we repeated the experiment after labeling cell surface proteins with biotin. DRM- and Triton X-100-soluble fractions were then pooled, and biotinylated proteins were isolated on streptavidin columns. SDS–PAGE and WB analysis revealed that DRM-associated N-cadherin is at least in part biotinylated demonstrating expression on the cell surface. Biotinylated TrR could be isolated only from Triton X-100-soluble fractions. The absence of TrR and the presence of cav-1 in total DRM-fractions confirmed that DRMs were correctly isolated (Fig. 6a, right panels). In agreement with this result, EGTA treatment of SBCl2 cells did not promote translocation of N-cadherin to DRMs (data not shown). Since the interaction with the actin cytoskeleton stabilizes cell–cell junctions and was shown to be required for concentrating N-cadherin in membrane rafts of myoblasts, we treated SK-Mel-28 cells with the F-actin depolymerizing agent cytochalasin D. We found that also actin cytoskeleton disruption did not perturb the interaction of N-cadherin with DRMs (Fig. 6b). In order to examine if membrane raft integrity is necessary for cell–cell junctions stability, we treated SBCl2, SK-Mel-28, and WM793 cells with MβC for times sufficient to dissociate N-cadherin from DRMs (Figs. 1c and 7a), and to extract most of cholesterol as monitored by staining with the cholesterol-binding drug filipin (Fig. 7b). Staining of cells with anti-N-cadherin and anti-β-catenin antibodies revealed that cell–cell adhesions containing both proteins were maintained in all cell lines, despite efficient cholesterol depletion and disruption of membrane rafts (Fig. 7b). We then asked if dissociation of N-cadherin from DRMs prevents its engagement in cell–cell junctions. We thus treated SK-Mel-28 cells with MβC alone for 60 min and in combination with EDTA during 5–10 additional minutes, in order to disrupt cell–cell adhesions and detach cells from the dish. The cells were then collected and plated on fibronectin-coated dishes or coverslips to promote attachment, in serum-free medium containing $CaCl_2$. After 75 min, most MβC/EDTA-treated and control cells (treated only with EDTA) were adherent and spread. Cells plated on fibronectin-coated dishes were subjected to DRM isolation, while those on coverslips were fixed and stained. We observed that in MβC/EDTA-treated cells replated on fibronectin N-cadherin was not associated with DRMs (consistently with the efficient cholesterol depletion as monitored by filipin staining; Fig. 8a,b), nevertheless, it was localized at cell–cell junctions (Fig. 8b). The same results were observed for WM793 cells (Supplementary Fig. 3).

All together these results indicate that in melanoma cells the association of N-cadherin with membrane rafts (1) is not dependent on cell–cell junctions assembly, nor on a stable actin cytoskeleton, thus indicating that it is not the consequence of its homophilic binding and clustering stabilized by cytoskeleton constraints;

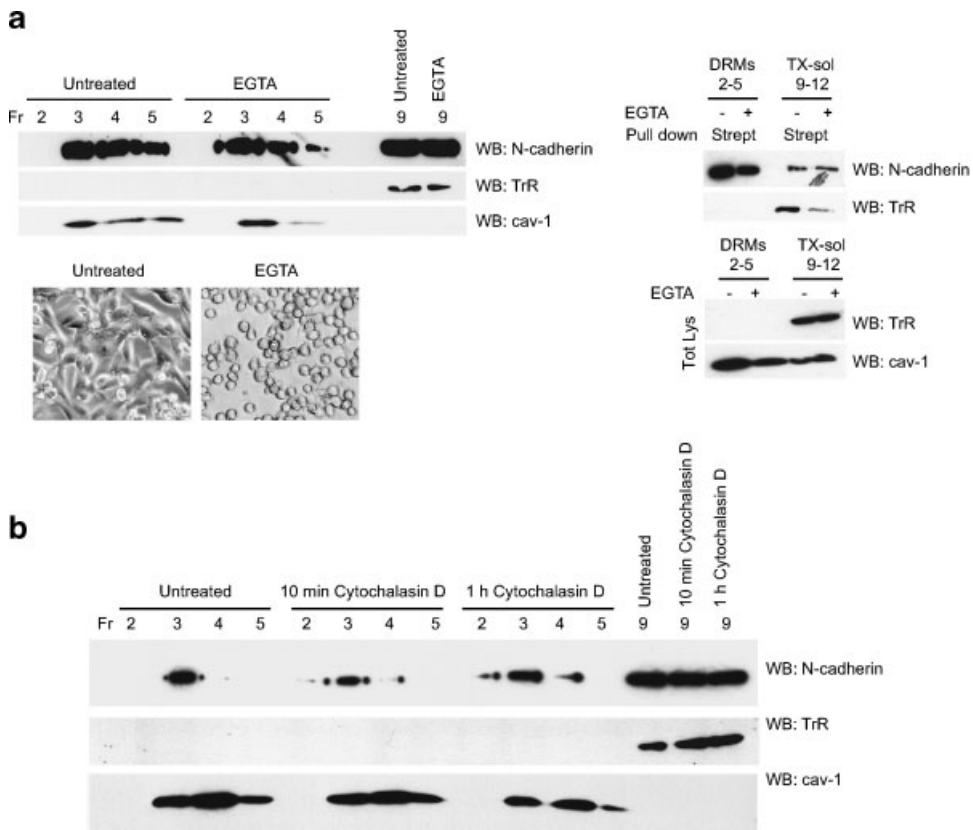

Fig. 6. Disruption of cell–cell junctions and of the actin cytoskeleton does not affect the association of N-cadherin with membrane microdomains. DRMs were isolated from SK-Mel-28 cells left untreated or treated with (**a**) 2.5 mM EGTA for 60 min, or (**b**) 2 μM cytochalasin D. The distribution of N-cadherin, TrR, and cav-1 in DRMs and in the Triton X-100-soluble fraction 9 was analyzed. In (a) right panel, isolation of biotinylated cell surface proteins revealed that a fraction of DRM-associated N-cadherin is at the cell surface also in EGTA-treated cells; (a) bottom panel, phase contrast pictures show the effect of EGTA on cell–cell adhesions and morphology.

(2) is not necessary for cell–cell junctions establishment and maintenance.

DISCUSSION

The phenomenon of cadherin switch, consisting in the upregulation of N-cadherin and the downregulation of E-cadherin expression levels, often occurs during the progression of several types of cancers, and it is usually associated with increased tumor invasion and metastatic abilities [Christofori, 2006]. In melanoma N-cadherin can promote malignancy by mediating the heterotypic adhesion between melanoma cells and fibroblasts or vascular endothelial cells, which facilitates dermal invasion and the interaction with the vasculature, promoting tumor cells dissemination through blood vessels. In addition, N-cadherin can also activate signaling pathways resulting in increased survival and motility, both directly and in cooperation with GF receptors [Li et al., 2003]. In this study, we provide evidence that in melanoma cells the function of N-cadherin may be regulated by an additional mechanism. Our results show that in some melanoma cell lines in vitro and in experimental melanomas in vivo a fraction of N-cadherin is targeted to cholesterol-sphingolipid-rich plasma membrane microdomains, where it interacts with β-catenin, p120-catenin, and possibly α-catenin. We also show that the localization of N-cadherin/catenins protein complexes in membrane microdomains is not dependent on the assembly of cell–cell junctions, and it is not necessary for cell–cell junction establishment and maintenance. On the one hand disruption of cell–cell adhesions or of the actin cytoskeleton does not affect the amount of N-cadherin associated to membrane microdomains, and on the other membrane microdomains disorganization and consequent dissociation of

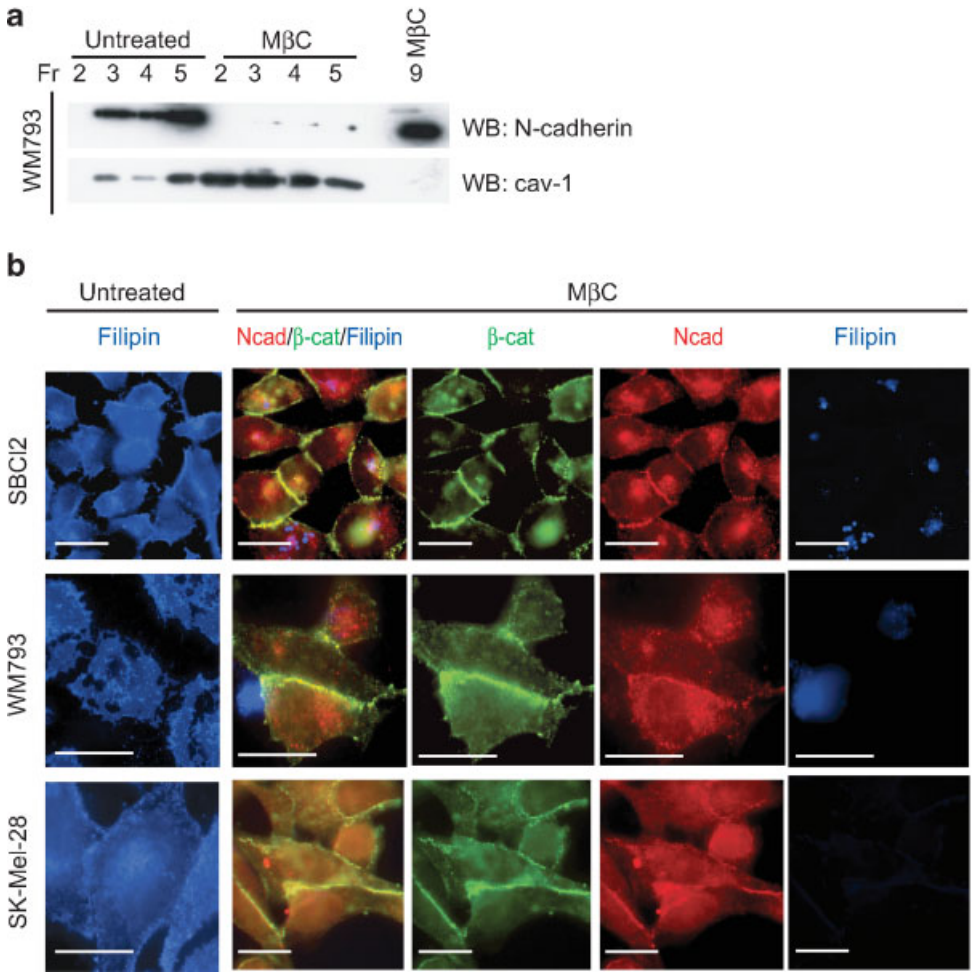

Fig. 7. Disruption of membrane microdomains does not impair the stability of N-cadherin-containing cell–cell junctions. **a**: Treatment of WM793 cells with 10 mM MβC caused N-cadherin dissociation from DRMs (isolated from 2 mg of total proteins). **b**: Cells were left untreated or treated with MβC. Panels in the left column show cholesterol distribution revealed by filipin staining in untreated cells; after cholesterol extraction, cell–cell adhesions containing both N-cadherin and β-catenin were maintained in all cell lines (right panels).

N-cadherin from this lipid environment does not impair cell–cell junctions stability or their assembly. This conclusion is further supported by the observation that SBCl2 and WM35 cells, in which N-cadherin association with membrane microdomains is not detectable, form N-cadherin-containing cell–cell adhesions. Importantly, the association of N-cadherin with membrane microdomains does not depend on N-cadherin expression levels: in fact N-cadherin was undetectable in DRMs of SBCl2, despite its high level of expression, comparable to that found in other cell lines here examined. These results reveal that two distinct pools of N-cadherin exist on melanoma cell surface: one pool is independent of lipid rafts and is engaged in cell–cell junctions, while a second pool is localized in membrane rafts and is not involved in cell–cell adhesion (Fig. 8c).

To our knowledge, this is the first time that a protein known to directly promote cancer progression is found to differentially partition in membrane rafts in cells derived from tumors of the same type, independently of its expression level. This observation suggests the existence of a mechanism driving N-cadherin to lipid rafts, which may be acquired by melanoma cells during malignant progression.

One question that immediately arose from this observation is whether the association of N-cadherin to membrane microdomains is regulated by signaling events known to promote melanoma progression. Treatment of melanoma

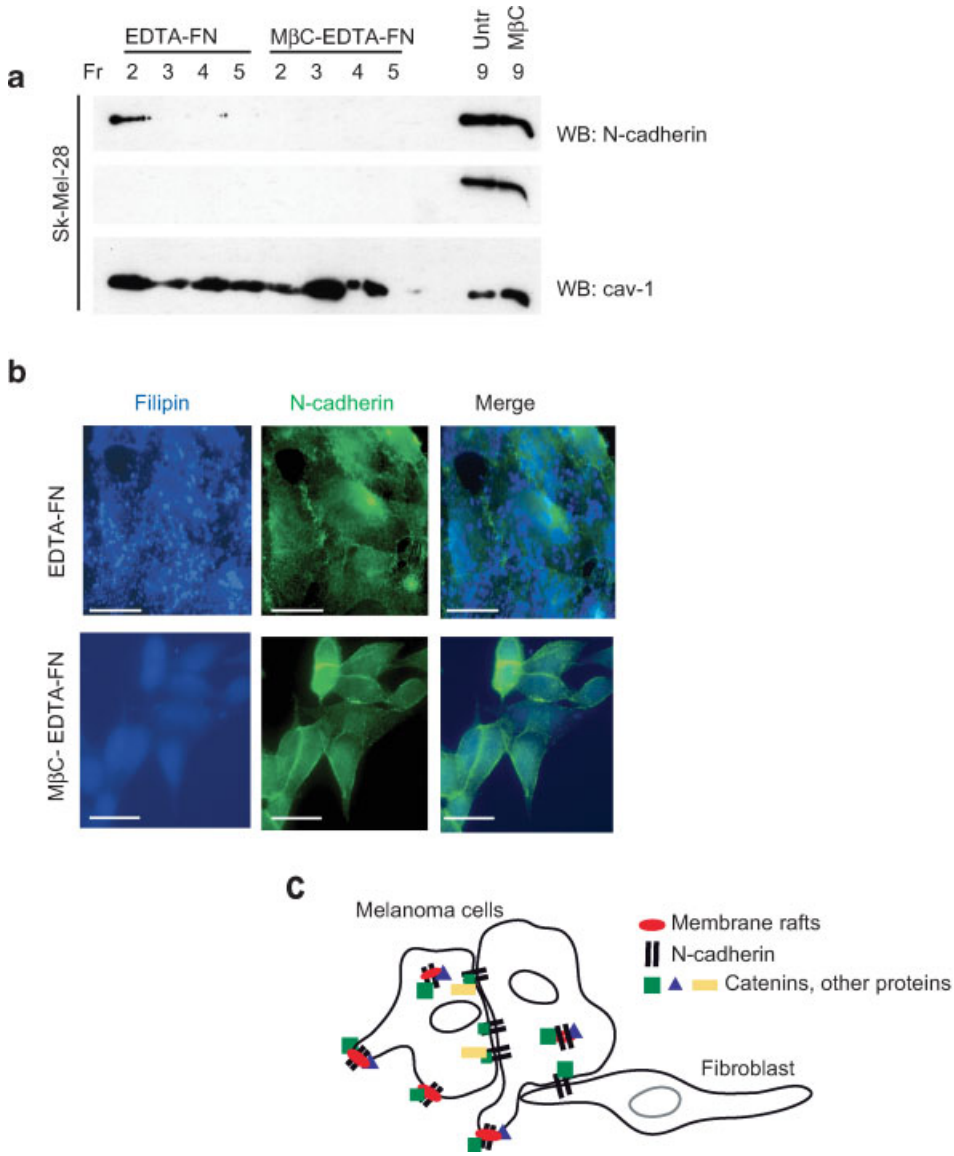

Fig. 8. Disruption of membrane microdomains does not impair the establishment of N-cadherin-containing cell–cell junctions. SK-Mel-28 cells were detached with EDTA as a control (EDTA-FN) or treated with MβC and detached with EDTA; cells were then replated on fibronectin-coated dishes or coverslips. After spreading cells were processed either for (**a**) sucrose gradient centrifugation or for (**b**) staining with filipin to visualize cholesterol, and with an anti-N-cadherin antibody to visualize cell–cell junctions. Bars, 50 μm. In (a) N-cadherin is still dissociated from DRMs (isolated from 1.8 mg of total proteins) in MβC-treated cells replated on FN. **c**: A model depicting N-cadherin distribution on the surface of melanoma cells. Two pools of N-cadherin are present at the plasma membrane of melanoma cells: one pool is engaged in cell–cell junctions and is connected to the actin cytoskeleton, the other pool is associated with membrane rafts outside cell–cell adhesions where it interacts with β-, p120-, and possibly α-catenin. Other proteins might physically and functionally interact with N-cadherin in membrane microdomains and modulate its function.

cells with serum, GFs, or chemicals stimulating or inhibiting these signaling pathways had no detectable effect on N-cadherin partitioning to lipid rafts. At this point, however, we cannot exclude that other signaling cascades might modulate N-cadherin–catenin targeting to lipid rafts, and more exhaustive studies may be warranted. It is known that during cell transformation plasma membrane lipid composition undergoes changes which can affect the formation and distribution of lipid rafts. In particular, increases in cholesterol and ganglioside levels, both constituents of lipid rafts, have been reported in solid tumors [Li et al., 2006]. In this respect, we have found no differences in the amount of cholesterol present in SBCl2 and

SK-Mel 28 cells (data not shown), but other sphingolipids and gangliosides might be differentially expressed in the two cell lines and affect N-cadherin interaction with membrane microdomains. It is possible that N-cadherin associates with membrane rafts directly, as a result of post-translational modifications such as glycosylation and acylation which would increase its affinity for raft lipids [Brown and London, 2000], or alternatively that it is driven to membrane microdomains by interaction with raft-resident proteins expressed or functional only in certain melanoma cells. In any case, the association could depend on biochemical features and mechanisms that are acquired by melanoma cells as a result of their progressive transformation.

The main outstanding question arising from these study concerns the biological significance of the association of N-cadherin and catenins with membrane microdomains. In contrast with primary myoblasts, the interaction with lipid microdomains is not necessary for the assembly and stability of cell–cell junctions in melanoma cells. This apparent divergence may be due, at least in part, to the fact that N-cadherin may perform unique functions in different cell types. In primary myoblasts N-cadherin is required to establish cell-to-cell adhesions, and its association with microdomain lipids and proteins might assure stability and functionality of these structures in this context. In transformed cells, like melanoma cells, N-cadherin is likely to perform more complex functions, being involved not only in the establishment of dynamic and heterotypic cell–cell adhesions with stromal fibroblasts or endothelial cells, but also in eliciting signaling events affecting survival and migration in a cell autonomous manner. In this respect, it was shown that in primary cerebellar neurons a fraction of N-cadherin is localized in membrane microdomains in the migrating growth cone, and the disruption of its association with DRMs prevents N-cadherin-dependent growth cone migration [Nakai and Kamiguchi, 2002]. The observation that in melanoma cells N-cadherin is complexed with catenins within microdomains, suggests a role in signaling, possibly in modulating pathways ultimately promoting cell malignancy, including the wnt pathway notoriously implicated in melanoma development [Weeraratna, 2005; Weeraratna et al., 2002]. The identification of other possible components of the N-cadherin–catenin complex interacting with rafts lipids may help characterize its function. We are currently addressing this question by performing comprehensive proteomics analysis of membrane rafts derived from melanoma cells at different stages of transformation. Another possibility is that DRM-localized N-cadherin undergoes raft-dependent endocytosis [Rajendran and Simons, 2005]. Endocytosis and recycling of N-cadherin and catenins could be a mechanism to ensure constant levels of these proteins in malignant cells, without the need to re-synthesize them; it is also possible that N-cadherin protein complex signals from endosomes.

In conclusion, we propose that the targeting of N-cadherin to membrane rafts provides melanoma cells with additional mechanisms to regulate the function of this molecule, and we suggest that aberrant protein targeting to membrane rafts may be a mechanism contributing to cancer progression. Elucidating the biochemical basis and the biological significance of N-cadherin association with membrane microdomains will be necessary in order to substantiate this hypothesis, and it may open new perspectives to better understand the role of this molecule in melanoma malignant progression.

ACKNOWLEDGMENTS

We are grateful to Dr. B. Giovanella, Dr. M. Herlyn, and Dr. D. Rimoldi for providing us with the melanoma cell lines. We also thank Dr. G.C. Alghisi and Dr. J. Zaric for discussions, Y. Monnier for discussions and help with the in vivo tumorigenesis experiments, and D. Germano for help in some experiments. Dr. S. Andrejevic-Blant and J.C. Stehle are acknowledged for help with the CD31 immunohistochemistry. Prof. F. Lejeune is acknowledged for continuous support. This work was supported by the Swiss National Science Foundation grant 3100A0-101739 to A.M. and by the MEDIC Foundation.

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