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DETERGENT-RESISTANT MEMBRANES-ASSOCIATION OF THE INTERLEUKIN-6 SIGNAL TRANSDUCER gp130 AND ITS MUTANT gp130LL/AA DIFFERS WITH THE METHODS OF ISOLATION

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Abstract

The transmembrane protein gp130 is the common signal transducer for the interleukin-6 (IL-6) type cytokines. It has been shown that cytoplasmic dileucine internalization motif also directs gp130 to the basolateral plasma membrane of polarized MDCK cells and protein partially localizes to detergent-resistant membranes (DRMs)/lipid rafts. Here we have studied whether the same dileucine motif is also raft association signal, similarly to sorting signals. Using two different lipid raft detergent-extraction protocols, we have found that wild type gp130, truncated gp130 lacking the cytoplasmic domain and gp130 mutant in which the di-leucine motif has been exchanged by two alanines have equal affinity for the liquid-ordered membrane domain independently of the methods of isolation. We demonstrate difference in cofractionation, fully or partially with lipid rafts of gp130wt and mutants depending on approach. Despite the differences, dileucine motif, which is both internalization and sorting one, is not a raft targeting signal.

Key words: gp130, MDCK, lipid rafts/DRMs

1. Introduction. Interleukin-6 (IL-6) cytokines belong to the haematopoietic cytokines [1]. IL-6 acts via a receptor complex consisting of an 80 kDa binding protein gp80, (IL-6R) and a 130 kDa signal transducer, gp130 [2, 3]. As common

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signal transducers of all IL-6 types of cytokines, gp130 molecules activate the Janus kinase/signal transducer and activator of transcription (Jak/STAT) cascade, thereby transducing the signal to IL-6 responsive genes in the nucleus [4].

Gp80 and gp130 are sorted to the basolateral plasma membrane in polarized epithelial Madin–Darby canine kidney (MDCK) cells. For gp80 two sequence motifs within the cytoplasmic domain are responsible for basolateral targeting, a tyrosine-based one (Y^{408}SLG) and a di-leucine type one ($L^{427}L^{428}$) [5]. Di-leucine internalization motif ($L^{786}L^{787}$) of gp130 is sufficient to mediate its basolateral sorting in MDCK cells [3].

Many proteins are associated with detergent-resistant membranes (DRMs)/ lipid rafts via different mechanisms [6, 7] and it is believed that lipid rafts are involved in signal transduction and protein sorting [7, 8]. Wild-type gp80 and gp130 are partially associated with lipid rafts in MDCK cells and raft localization must be a genuine property of each receptor variant [2, 9]. Due to their physicochemical properties, lipid rafts can be isolated with non-ionic detergents (Triton X-100, Brij 58) at 4 °C as low density, detergent-resistant membranes (containing lipid rafts and caveolae) on sucrose density gradients [10] or directly separated from non-DRMs with non-gradient isolation [11].

In this study, we compared the association of gp130wt (wild type), gp130ΔCD (lacking the cytoplasmic domain) and gp130LL/AA (di-leucine sorting motif is exchanged by two alanines) with DRMs in polarized MDCK cells using two different protocols [11, 12] and demonstrated partial or complete lipid raft targeting. There is not difference in gp130LL/AA lipid raft affinity compared with gp130wt and gp130ΔCD independently of the method. Our findings show that di-leucine internalization and basolateral sorting motif is not raft targeting signal, suggesting that internalization and sorting of gp130 in MDCK cells and raft association are two processes requiring different signals.

2. Materials and methods. ***2.1. Materials.*** All chemicals were supplied by Sigma–Aldrich/Fluka (Taufkirchen, Germany) unless otherwise indicated. Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), bovine serum albumin (BSA) and G418 sulphate were purchased from PAA (Gölbe, Germany). Fetal calf serum (FCS), penicillin/streptomycin and trypsin/EDTA were obtained from Biochrom (Berlin, Germany). Antibodies were obtained as follows: mouse IgG1 α-gp130 (clone BP-4) from Diaclone (Besançon, France), rabbit α-caveolin-1 (N-20) from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), HRP-linked α-mouse IgG and HRP-linked α-rabbit IgG from Amersham Pharmacia Biotech (Freiburg, Germany). ECL western blotting detection system was purchased from Amersham Pharmacia Biotech (Freiburg, Germany).

2.2. Cell culture and transfection. MDCK cells, strain II were maintained and transfected as previously described [3]. For raft association studies, cells were plated and grown for four days on 60 mm and 100 mm cell culture dishes.

2.3. Sucrose-gradient isolation of detergent-resistant membranes (DRMs). To isolate DRMs of polarized MDCK cells stably transfected with gp130wt and gp130LL/AA, cells were grown to confluency for four days. DRMs were obtained according to an established protocol [12]. Briefly, cells were scraped into ice-cold TNE buffer (25 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100 and protease inhibitors. Cells were homogenized, adjusted to 40% sucrose (in TNE), and overlaid with 30% and 5% sucrose. Samples were ultracentrifuged in a Beckman SW40 rotor at 37 000 rpm for 17 h at 4 °C. Fractions (1 ml) were harvested, and proteins were precipitated with trichloroacetic acid and analyzed by Western blotting.

2.4. Non-gradient isolation of detergent-resistant membranes (DRMs). Polarized MDCK cells stably transfected with gp130wt, gp130ΔCD and gp130LL/AA were grown to confluency for four days. Cells were washed with PBS buffer containing protease inhibitors on ice and lysed for 30 min at 4 °C in 1.5 ml of Hepes lysis buffer (250 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100 and protease inhibitors) on shaker (10 rpm). Cells were scribed, homogenized and ultracentrifuged at 36 000 rpm for 30 min at 4 °C in an Ultrazentrifuge Sorvall Combi Plus OTD-C+. Supernatants representing the soluble material were separated from pellets that were solubilized in 1.5 ml of solubilization buffer (50 mM Tris-HCl, pH 8.8, 5 mM EDTA, 1% SDS). Proteins were recovered by trichloroacetic (TCA) acid precipitation, solubilized in Laemmly buffer and analyzed by Western blotting [11].

2.5. Western blotting and immunodetection. Electrophoretically-separated proteins were transferred to polyvinylidene difluoride membranes by the semi-dry western blotting method. Non-specific binding was blocked with 5% skimmed milk powder in TBS-T (0.1% Tween 20) for 1 h. Blots were incubated with human gp130-specific mAb (clone BP-4) and respectively with a caveolin-1-specific rbAb followed by incubation with HRP-conjugated goat anti-mouse and anti-rabbit IgG for 1 h. The immunoblots were developed using the enhanced chemiluminescence (ECL) system according to the manufacturer's instructions.

3. Results. Gp130 is a predominantly basolateral-targeted protein based on di-leucine sorting signal within the cytoplasmic domain. Its partial lipid raft localization has been demonstrated when a cold Triton X-100 detergent has been used [2, 3, 5]. In order to analyze the correlation between lipid raft association and sorting of gp130 (whether the $L^{786}L^{787}$ sorting motif is also a raft-assosiation motif), we used two different raft isolation approaches.

DRMs from gp130wt and gp130LL/AA stably-transfected MDCK cells were subjected to sucrose density gradient centrifugation, according to an established protocol [2, 9, 12]. Nine fractions (from 4 to 12) were harvested, and proteins were analyzed by Western blotting. Previously, it was shown that fractions 1, 2 and 3 did not co-float with scaffolding protein caveolin-1 as lipid raft marker and gp130 proteins were not detected in these fractions [9]. Fractions from 4 to 12

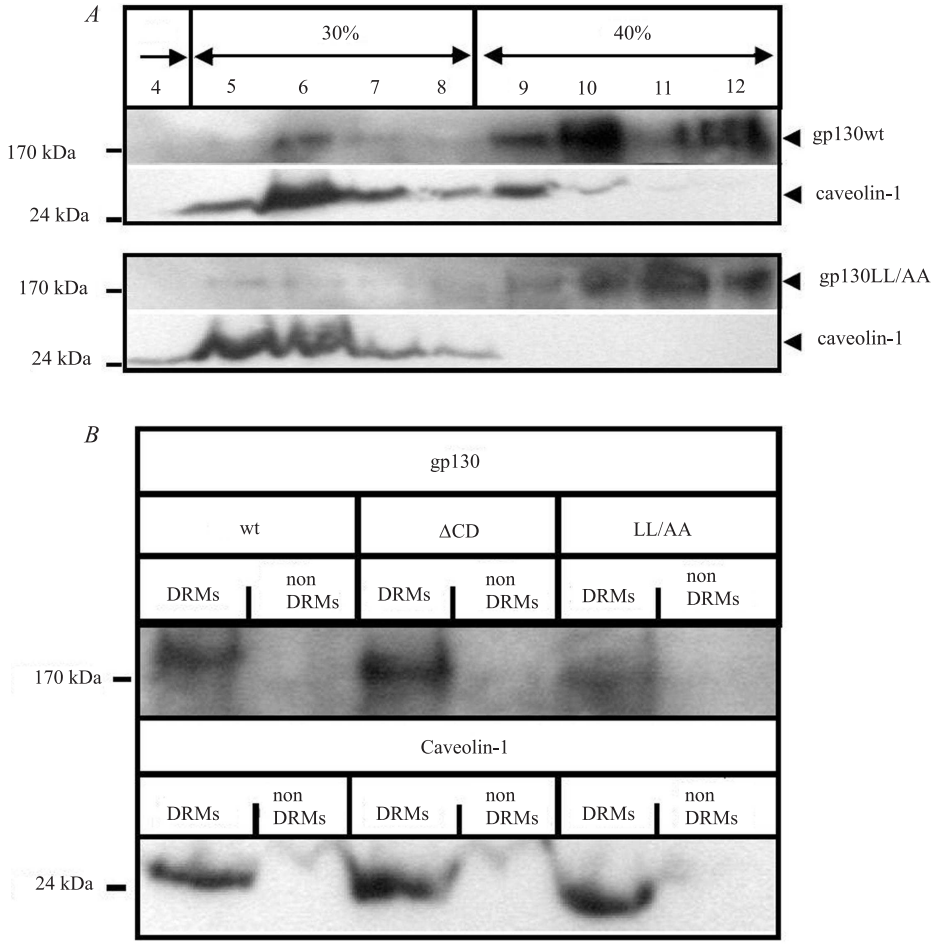

Fig. 1. Differences in association with DRMs/lipid rafts of human gp130wt and mutants in stably transfected polarized MDCK cells. Caveolin-1 is used as a raft-marker. *A*) Partial association with DRMs/lipid rafts of human gp130wt and gp130LL/AA after sucrose-gradient isolation. *B*) Complete association with DRMs/lipid rafts of human gp130wt, gp130LL/AA and gp130ΔCD after non-gradient isolation

were analyzed (Fig. 1*A*) and caveolin-1 floated mainly to fractions 4–7 (lipid raft fractions) corresponding to the 5%/30% sucrose interface. The vast majority of gp130wt and gp130LL/AA proteins were found in the bottom corresponding to the 30%/40% interface as non-raft fractions (fractions 8–12). A minor amount was present in fractions 4–7 co-floating with caveolin-1. The same flotation was found for gp130ΔCD proteins lacking the cytoplasmic domain [9]. This demonstrates that $L^{786}L^{787}$ sorting motif of gp130 is not raft association signal even when gp130 proteins are partially associated with lipid rafts.

To corroborate this finding, we used a Slimane and colleagues protocol [11], where DRMs and non-DRMs are separated by non-gradient centrifugation

(Fig. 1*B*). Gp130wt and mutants showed an equal distribution to DRMs and the majority of proteins were preferentially labelled to DRMs co-fractionated with caveolin-1 and almost nothing was labelled at non-DRMs fractions. Gp130LL/AA and gp130ΔCD did not show different affinity to Triton X-100-resistent membranes compared to gp130wt (Fig. 1*B*), confirming that di-leucine sorting motif is not a raft association signal.

4. Discussion. In the present study, the role of the di-leucine ($L^{786}L^{787}$) basolateral sorting signal within the cytoplasmic domain of gp130 for its raft association in polarized MDCK cells was analyzed.

Previously, it was shown that gp80 (IL-6-R) and its signal transducer gp130 were mainly located at the basolateral plasma membrane [5]. The other members of the IL-6 receptor family were apical (ciliary neurotrophic factor receptor, CNTF-R) or non-polarized (leukaemia inhibitory factor receptor, LIF-R) distributed although they all needed to associate with gp130 for signalling to occur [2]. CNTF-R co-fractionated quantitatively with lipid rafts independently of the approach. In contrast, gp130 and LIF-R were found to associate with lipid rafts only partially when detergents were used for isolation [2]. Wild-type gp80 is also partially associated with lipid rafts in MDCK cells [9]. This association is in dependence of its cytoplasmic domain. Gp80 mutants which lack parts of the cytoplasmic domain (lack of $L^{427}L^{428}$ or both $L^{427}L^{428}$ and Y^{408}SLG) are more apically expressed than the wild-type and show an increased affinity for detergent-resistant membranes (DRMs, lipid rafts) [9]. Raft association of gp130 was also studied with respect to sorting. Human gp130 was preferentially labelled at the basolateral membrane of transfected MDCK cells, whereas in cells that express gp130ΔCD and gp130LL/AA a complete reversal of polarity (at the apical membrane) was observed [3]. Partial association and almost equal affinity of gp130wt and gp130LL/AA with DRMs (when Triton X-100 and sucrose gradient centrifugation are used, Fig. 1*A*) exclude di-leucine sorting signal as a raft targeting signal. The majority of these proteins were found in non-raft fractions (8–12) and the minority – in raft fractions (4–7). The same was found for gp130ΔCD [9]. But we observed complete raft association of gp130wt, gp130LL/AA and gp130ΔCD using non-gradient approach of DRMs/non-DRMs separation. The majority of the proteins after separation with this approach co-floated with the majority of caveolin-1 (raft fractions), and gp130LL/AA did not show a different affinity to Triton X-100 resistant membranes than gp130wt and gp130ΔCD (Fig. 1*B*), suggesting that raft association signals are not determined within cytoplasmic domain of the proteins.

5. Conclusions. Our data indicate that despite the differences observed by two methods, di-leucine sorting motif of gp130 is not raft association signal, confirming previous findings that raft association of gp130 is independent of its polarity.

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