Lipid rafts couple store-operated Ca$^{2+}$ entry to constitutive activation of PKB/Akt in a Ca$^{2+}$/calmodulin-, Src- and PP2A-mediated pathway and promote melanoma tumor growth

Shlomit Fedida-Metula$^{1,2,3}$, Ben Feldman$^{1,4}$, Valeria Koshelev$^{3}$, Uliana Levin-Gromiko$^{5}$, Elena Voronov$^{2}$ and Daniel Fishman$^{1,*}$

$^{1}$Department of Morphology, $^{2}$The Shraga Segal Department of Microbiology and Immunology, $^{3}$Department of Virology and Developmental Biology and $^{4}$Department of Physiology, Faculty of Health Sciences, Ben-Gurion University Cancer Research Center, Ben-Gurion University of the Negev, PO Box 653, Beer-Sheva 84105, Israel

$^{*}$To whom correspondence should be addressed. Tel: +972 8 6477319; Fax: +972 8 6477627; Email: dmif@bgu.ac.il

Central role of constitutively active protein kinase B/Akt (PKB) in melanoma drives the search for new targets to abolish its deranged signaling. PKB activation is promoted by cholester-enriched lipid rafts and is Ca$^{2+}$-dependent, but the pathway linking rafts and Ca$^{2+}$ to deregulation of this enzyme remains poorly understood. Here employing B16BL6 melanoma model, we show that ablation of rafts with methyl-$\beta$-cyclodextrin (M$\beta$CD) inactivated PKB by inhibiting Src kinase and reactivating the negative PKB modulator, PP2A phosphatase. Blockade of PP2A with okadaic acid rescued PKB, indicating that raft ablation reactivated PP2A through inhibiting Src. Indeed, direct Src blockade with the Src kinase inhibitor-1 or the dominant-negative Src-mutant was sufficient for PP2A reactivation and downregulation of PKB, whereas reconstitution of rafts in M$\beta$CD-treated cells restored PKB, PP2A and Src activities to their basal levels. This pathway was also interrupted by inhibition of the Ca$^{2+}$ sensor calmodulin, either by its antagonist N-(6-amino-hexyl)-5-chloro-1-naphtalenesulfonamide or the Ca$^{2+}$-insensitive calmodulin-mutant or the intracellular Ca$^{2+}$-chelator 1,2-bis(o-aminoophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra-(acetocetylnyl)-ester or by diminishing the store-operated Ca$^{2+}$ entry with 2-aminoethoxydiphenyl borate or small hairpin RNA against Stim1. Ablation of rafts prevented Stim1-mediated store-operated Ca$^{2+}$ entry, aborted Ca$^{2+}$$^{2+}$ stimulation of raft-residing calmodulin and disrupted its Ca$^{2+}$-dependent binding to Src, abolishing Src activity and entire Src/PP2A/PKB cascade. Most importantly, blockade of this cascade in the tumor site by raft-ablating M$\beta$CD, administered to melanoma-bearing mice, robustly retarded tumor growth and extended animal survival. Together, our data suggest that lipid rafts couple store-operated Ca$^{2+}$ entry to sustained activation of major tumor-promoting signaling elements in melanoma cells and underscore the potential of raft-targeting agents as effective anticancer drugs.

Introduction

Malignant melanoma is the deadliest form of skin cancer and, when disseminated, associates with median survival time of $\sim$6 months. The invasive growth of this tumor, apoptosis resistance and metastasis are promoted by multiple signaling abnormalities, of which the constitutive activation of protein kinase B/Akt (PKB) occurs in $\sim$50% of cases, correlating with poor prognosis in patients (1,2). Normally, activation of PKB through phosphorylation in a phosphatidylinositol-3 kinase (PI-3K) pathway is counterbalanced by the phosphatase and tensin homologue on chromosome 10 lipid phosphatase and protein phosphatases PP2A and PP1, which interfere with membrane PKB trafficking and remove activating phosphates, respectively (1-3). Melanoma cells frequently fail to maintain this mechanism due to the loss of phosphatase and tensin homologue on chromosome 10, Ras oncogene mutations or amplification of receptor tyrosine kinase and PKB isoform-encoding genes (1-5). Once deregulated, PKB elicits its tumor-promoting function in cooperation with B-Raf oncogene (1,2,6,7) but importantly overrides melanoma-suppressive effect of pharmacological B-Raf blockers (4,5,7,8) that highlights the central role of this enzyme in melanoma malignancy. To this end, no clinical PKB inhibitors are available and identification of new targets to abolish its deranged signaling is in the focus of intense research (1).

Cholesterol-enriched lipid raft membrane microdomains now emerge as major cellular signal nodes that promote interactions between discrete signaling intermediates through selective recruitment of specific subsets of membrane-associated proteins (9). Quantitative and qualitative raft changes due, in part, to abnormal accumulation of cholesterol by malignant cells have been identified in many cancer types including melanoma and implicated in signaling deregulations that enhance tumor growth, survival and invasion (9-12). Regarding the PKB, it was proposed that increased cholesterol content expands and/or modifies cancer raft microdomain platforms, facilitating re-uptake of receptor tyrosine kinases, focal adhesion elements, PI-3K subunits, other PKB activators or PKB itself, capable of recruitment to the rafts by growth factor or cytoadhesion stimuli (9-16). Indeed, PKB is extremely sensitive to disintegration of cholesterol-enriched rafts (16,17) but the mechanism of its raft-dependent deregulation in melanoma remains poorly understood. Recently, we reported that this PKB activity is promoted by cytosolic Ca$^{2+}$, [Ca$^{2+}$]$_{cyt}$, in a pathway that involves accelerated Ca$^{2+}$ influx via the store-operated Ca$^{2+}$ channels (SOC) and the Ca$^{2+}$ sensor calmodulin (17,18). Calmodulin couples [Ca$^{2+}$]$_{cyt}$ to the downstream signaling events by interacting with its multiple effector proteins. Examples relevant for PKB signaling include PKB, PI-3K, calmodulin-dependent, focal adhesion and Src kinases (19-22), but of these proteins, only Src permanently resides in the rafts of malignant melanoma cells (23). Src activates PKB via PI-3K (24) or through phosphorylation-induced inhibition of PP2A (25), the dysfunction of which was reported in melanoma (26) and other tumors (3) displaying elevated basal Src and PKB activities. Notably, lipid rafts have been implicated in modulation of SOC, as their major regulator Stim1 could be targeted to these microdomains, where it interacts with channel-constituting TRPC and/or Orai proteins and triggers Ca$^{2+}$ entry (27-29). However, the networks linking SOC and Ca$^{2+}$/calmodulin to raft-dependent constitutive PKB activation are unknown. Here employing B16BL6 melanoma model, we show coupling of store-operated Ca$^{2+}$ influx to raft-residing calmodulin that promotes Ca$^{2+}$-driven binding of calmodulin to Src, sustains Src activity and Src-induced inactivation of PP2A. Interruption of this mechanism, both in vitro and in vivo, with the cyclic oligosaccharide methyl-$\beta$-cyclodextrin (M$\beta$CD), which ablates rafts by extracting membrane cholesterol (30), inhibits PKB by reactivating PP2A, retards melanoma tumor growth and prolongs survival of melanoma-bearing mice.

Materials and methods

Cell culture and reagents

B16BL6-8 melanoma cells were described previously (15,16) and kindly provided by Prof. E.Gorelik (Department of Pathology, University of Pittsburgh...

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Cancer Institute, University of Pittsburgh, Pittsburgh, PA). Cells were cultured in RPMI medium supplemented by antibiotics and 10% bovine serum (Biological Industries, Beth Haemek, Israel). For cholesterol depletion/replenishment experiments, cultures were maintained in serum-free conditions. MJCD, water-soluble cholesterol and filipin were purchased from Sigma (Rochester, NY). p85 PI3-K antibodies from Cell Signaling (Beverly, MA); anti-actin from MP Biomedicals (Aurora, OH); Fluo-4-AM and Alexa633-conjugated CTX from Invitrogen, Molecular Probes (Eugén, OR) and protein A sepharose from Amersham Biosciences (Uppsala, Sweden). K295R p60 Src encoding plasmid vector (31) was provided by Pro. A.Rao (Harvard Medical School and the Center for Blood Research Institute for Harvard, MA). Small hairpin RNA (shRNA) encoding (T33619A) or control (TR003) plasmids were purchased from Origene (Rockville, MD). Transfections were carried out using Cellfectin reagent (Open Biosystems, Huntsville, AL) according to the protocol provided by the manufacturer. In most of experiments, 1 µg of plasmid was used to transfect cells and plated onto six-well plates (1.2 x 10^5 cells per well). For raft isolation studies, 10 µg of Stim1 shRNA-encoding vector were transfected into cells and plated onto 100 mm culture dishes (1.5 x 10^5 cells per dish). In experiments exploiting Stim1-YFP, 0.2 µg DNA were transfected into cells, plated onto Nunc-chambered cover-glass (Nunc A/S, Roskilde, Denmark) (0.3 x 10^5 cells per chamber). In all experiments, cells were analyzed 48 h post-transfection.

Preparation of total protein extracts, immunoprecipitation and western blotting analysis
Total protein extracts were prepared using a buffer (10 mM Tris–HCl, pH 8.0, 1% vol/vol sodium dodecyl sulfate) preheated to 100°C and cells homogenized by repeated passage through a syringe equipped with 27 gauge needle. Debris was removed by centrifugation at 12 000 g for 5 min at room temperature. For immune precipitation, cells were lysed using a buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate and protease inhibitors), homogenized and debris removed by centrifugation (12 000 g, 15 min, 4°C). The resulting clear supernatants (500 µg of protein in 0.2 ml of lysis buffer) were incubated with anti-calmodulin antibodies (diluted 1:33) and protein A sepharose (Amersham Biosciences) on rocking platform (12 h, 4°C). Total cell protein samples or immune complexes were then harvested by adding dodecyl sulfate-polyacrylamide gel electrophoresis samples and subjected to western blotting analysis with anti-pAkt, anti-pPP2Ac or anti-pSrc antibodies (1:50, each at 2 µg/ml). Blots were incubated with Alexa633-conjugated CTX (1 µg/ml) in CaCl2-supplemented (2 mM) Ringer’s solution for 30 min on ice and washed three times with Ca2+-free Ringer’s solution. Ca2+-free Ringer’s solution preheated to 37°C was added to cultures and cells stimulated with 2 µM Fluorescein isothiocyanate dye. Images were processed with FlowJo software.

Animal studies and immunohistochemistry
All in vivo studies were approved by the Institutional Committee for Ethical Care of Laboratory Animals (Ben-Gurion University of the Negev). To generate experimental tumors, B16BL6-8 cells (2.5 x 10^6 cells in 0.3 ml of sterile saline) were inoculated into the footpad of 8-week-old C57BL/J mice (Harlan, Jerusalem, Israel). 300 mg/kg of MJCD in 0.3 ml of sterile saline were weekly intraperitoneally administered. First injection was applied at day 8 after tumor cell implant. Melanoma specimens were fixed in 4% paraformaldehyde, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Antigen retrieval protocols included boiling for 20 min in 20 mM citrate buffer solution (pH 6). Four micron sections of the plugs were subjected to immunohistochemical analysis with anti-pAkt, anti-pPP2Ac or anti-pSrc antibodies (1:50, each at 4°C overnight). Staining intensity was assessed using ImageJ software.

Statistical analysis
All analyses were performed using the SPSS software. Student’s t-test was used for calculation of significance in all in vitro studies and for estimating differences of tumor sizes between MJCD-treated and control animals. The Kaplan–Meir test was used for statistical analysis of animal survival.

Results
Ablation of rafts inactivates PKB by eliminating Src-induced inhibitory phosphorylation of PP2A phosphatase
Previously, we reported that ablation of cholesterol-enriched rafts in melanoma cells abolishes aberrantly high basal PKB activity (37). Since raft-residing Src kinase is frequently upregulated in melanoma tumors (23,35) and might elicit inhibitory phosphorylation of the catalytic PP2A subunit (PP2Ac), we have first asked whether alteration of raft integrity inactivates PKB by inhibiting Src and eliminating Src-induced PP2A repression. As activation of PKB and Src and inactivation of PP2A are faithfully reflected by their phosphorylation status (2,25,36), we have used western blotting analysis to monitor levels of Ser473-phosphorylated PKB (active, pPKB), Y407-phosphorylated PP2A-C (inactive, pPP2A) and Y416-phosphorylated Src (active, pSrc) in B16BL6-8 melanoma cells treated with the raft-ablating agent MJCD. Detectable basal levels of all three phosphoproteins were induced by MJCD treatment. MJCD reduces the levels of Ser473-phosphorylated PKB and Y407-phosphorylated PP2A, indicating that Src-induced PP2A repression is important for PKB activation by melanoma cells. Since the total levels of Src in melanoma cells are not substantially reduced by MJCD treatment (23,35), these results suggest that raft-residing Src kinase is a critical regulator of PKB activation in melanoma cells.
**Fig. 1.** Ablation of rafts inactivates PKB by eliminating Src-induced inhibitory phosphorylation of PP2A phosphatase. (A) Western blotting analysis of pAkt, pPP2Ac, pSrc, Akt1, PP2Ac and Src expression in B16BL6-8 cells treated by indicated concentrations of MβCD for depicted time intervals. (B) MβCD-treated cells (14 mM, 2 h) were maintained for additional 1 h in serum-free medium in the presence water-soluble cholesterol (chol: 25 and 50 μM, 1 h) and subjected to
were identified, whereas application of MJCD (7 and 14 mM, 1 and 2 h) dose- and time-dependently reduced pAkt, pPP2Ac and pSrc but not their total protein expression (Figure 1A). To confirm that observed hypophosphorylation was specifically triggered by disintegration of cholesterol-enriched rafts, pAkt, pPP2Ac and pSrc were assessed after reconstitution of these microdomains in MJCD-treated cells achieved by incubating these cells in the culture medium supplemented with water-soluble cholesterol (20 and 50 µg/ml, 1 h). The cholesterol repletion procedure restored cellular cholesterol content and membrane expression of the raft-marker GM1 ganglioside, which were decreased by MJCD, pointing to lipid raft recovery, as determined by flow cytometry with the cholesterol and GM1 probes filipin and CTXB (34), respectively (Supplementary Figure 1A is available at Carcinogenesis Online). Reconstitution of rafts also restored pAkt, pPP2Ac and pSrc expression to levels detected in the control cells (Figure 1B), indicating that raft ablation alters PKB, PP2A and Src activities in reversible manner. Remarkably, reactivation of PP2A and inactivation of PKB (but not of Src) were prevented by the PP2A inhibitor okadaic acid (10 and 100 nM) applied in the course of MJCD treatment (Figure 1C). In contrast, the blocker of related PKB-inhibitory phosphatase PP1 tautomycin (3 and 300 nM) produced minimal effect on PP2A and PKB (Figure 1D). These data suggested that PK2A was reactivated through inhibition of Src and, indeed, direct Src blockade with the membrane permeable Src kinase inhibitor-1 (10 µM, 10 h) was sufficient to diminish expression of pSrc, pPP2Ac and pAkt (Figure 1E). To verify the results of pharmacological Src inhibition, we interfered with Src activity by overexpressing its dominant-negative mutant, deficient for adenosine triphosphate binding due to the K295R substitution (K295R p60Src) (31). Transient transfection of the K295R p60Src-encoding vector triggered a ~10-fold reduction of pSrc, pPP2Ac and pAkt levels relative to the control samples (Figure 1F). Hence, in these malignant cells, raft-dependent constitutive activation of PKB is sustained through the double-negative mechanism via Src and PP2A, whereas blockade of Src, both directly and by raft ablation, reactivates PP2A and abolishes PKB activity.

Ca²⁺-dependent binding of the Ca²⁺ sensor calmodulin to raft-residing Src sustains activation of Src, Src-induced inhibition of PP2A and thereby PKB activity

Previous studies have shown that high basal PKB activity in melanoma can be reverted by reducing the [Ca²⁺]cyt with the membrane-permeable Ca²⁺ chelator BAPTA-AM or by the calmodulin antagonist W7 (17). To determine whether this Ca²⁺/calmodulin-dependent PKB activation is mediated by Src through inhibiting PP2A, we have tested the effects of W7 and BAPTA-AM on pAkt, pPP2Ac and pSrc expression levels. Application of W7 to B16BL6-8 cells (30 and 50 µM, 1 and 3 h) dose- and time-dependently diminished levels of all three phosphoproteins (Figure 2A). Notably, pAkt and pPP2Ac were rescued by addition of okadaic acid (Figure 2B) but not of tautomycin (Figure 2C) to W7-treated cells. Similar reduction of pAkt, pPP2Ac and pSrc was detected in BAPTA-AM-treated cells (20 or 50 µM, 1 and 3 h), whereas okadaic acid (Figure 2B) but not tautomycin (Figure 2F) restored expression of pAkt and pPP2Ac to their basal levels. We also studied the Ca²⁺-dependent function of calmodulin by overexpressing its mutant, incapable of Ca²⁺-binding due to the alanine-to-aspartate substitutions in four EF hand motifs of this protein (CaM1,2,3,4) (32). Transient transfection of CaM1,2,3,4-encoding vector produced a ~5-fold reduction of pAkt, pPP2Ac and pSrc compared with the cells transfected with the control plasmid, whereas overexpression of WT-CaM was followed by a ~1.5- to 2-fold elevation in these phosphoproteins (Figure 2G). Since both raft ablation and inhibition of Ca²⁺/calmodulin abolished PKB activation by eliminating Src-induced PP2A blockade, it was then tested whether calmodulin compartmentalizes in lipid raft microdomains, where it may bind Src, other PKB-regulatory molecules or PKB itself and modulate their activities. Raft extract resistance with the cold detergent Triton X-100 and have high lipid to protein ratio, which permits their isolation because of increased buoyancy in sucrose density gradients (9). We took advantage on these properties and assessed partition of calmodulin, Src, PP2A, p85 regulatory PI-3K subunit, PKB1 and PKB in the low-density (raft-enriched) versus high-density (non-raft) cell fractions prepared from B16BL6-8 cells. Identification of raft fractions in the sucrose gradient was performed using the GM1 probe CTXB and dot blot analysis. Of the aforementioned proteins, only calmodulin and Src were coexpressed with GM1 in the low-density raft fractions 4 and 5 (Figure 2A). Disintegration of rafts by MJCD (14 mM, 2 h) was manifested by GM1 relocation to the high-density fractions and also led to accumulation of calmodulin and Src in the latter fractions (Figure 3B). Replenishing MJCD-treated cells with cholesterol (50 µg/ml, 1 h) restored the original distribution of all three molecules in the sucrose gradient (Figure 3C) that confirmed their specific association with cholesterol-enriched rafts. BAPTA-AM and W7 treatments (50 µM, 3 h each) preserved lipid raft integrity, since GM1 was retained in fractions 4 and 5 (Figure 3D and E, respectively). The latter fractions isolated from BAPTA-AM-treated cells contained calmodulin and Src (Figure 3D), indicating that partition of these proteins in the rafts does not require Ca²⁺. In contrast, W7 shifted calmodulin to the high-density fractions but did not markedly alter partition of Src in the rafts (Figure 3E). The results of fractionation analysis implied that lipid rafts promote Ca²⁺-dependent interactions between raft-residing calmodulin and Src. To test this hypothesis, we conducted immune precipitation experiments and, indeed, pulled down Src with calmodulin-reactive antibodies in the control but not in MJCD-treated cells (Figure 3F). The binding of calmodulin to Src was fully restored after cholesterol repletion of the latter cells (Figure 3F). Similarly, precipitation of Src with calmodulin-reactive antibodies was inhibited by W7 and BAPTA-AM (Figure 3F) and also abolished by overexpression of CaM1,2,3,4 but not of WT-CaM (Figure 3G). Thus, activation of Src, triggering the Src/PP2A/PKB cascade, is sustained by its Ca²⁺-driven binding to calmodulin in melanoma rafts and aborted when interactions between both proteins are disrupted by calmodulin blockade or alteration of raft integrity.

Lipid rafts couple store-operated Ca²⁺ influx to Ca²⁺/calmodulin and the downstream Src/PP2A/PKB cascade

Raft-localized calmodulin might be stimulated by SOC, the robust function of which sustains PKB activity in malignant melanoma cells (17,18). Since activation of SOC in the rafts could be triggered by Stim1 recruited to and clustered in these microdomains in response to depletion of intracellular Ca²⁺ stores (26–28,33), we assessed the effect of MJCD on formation of peripheral Stim1 aggregates, partition of Stim1 in the rafts and [Ca²⁺]cyt with the membrane-permeable Ca²⁺ sensor calmodulin to raft-residing Src sustains activation of Src, Src-induced inhibition of PP2A and thereby PKB activity.
Inhibition of Ca\(^{2+}\)/calmodulin abolishes Src-induced inhibition of PP2A and thereby high basal PKB activity. Western blotting analysis of pAkt, pPP2Ac, pSrc, Akt, pPP2Ac and Src expression in B16BL6-8 cells treated by W7 (A) or BAPTA-AM (D). Indicated concentrations of okadaic acid (OA) (B and E) or tautomycin (Tm) (C and F) were applied in the course of W7 or BAPTA-AM treatments (50 \(\mu\)M, 3 h each). (G) pAkt, pPP2Ac, pSrc, Akt, pPP2Ac and Src expression was analyzed in cells transiently transfected with pcDNA3-WT-CaM- and pcDNA3-CaM\(_{1,2,3,4}\)-encoding vectors and pcDNA3 control plasmids. (A–G)
fractions (Figure 4B) and elicited SOC-mediated Ca\(^{2+}\) influx (Figure 4C) in the control but not M\(\beta\)CD-treated cultures. The cholesterol replenishment of the latter cultures recovered formation of Stim1 clusters, restored the original pattern of Stim1 in the raft versus non-raft fractions and reverted inhibition of the store-operated Ca\(^{2+}\) entry (Figure 4A–C), confirming that lipid raft integrity determines SOC function in these malignant cells. If raft ablation interferes with Ca\(^{2+}\) stimulation of calmodulin through inhibition of SOC, then direct SOC blockade should downregulate the Src/PP2A/PKB pathway without excluding calmodulin and Src from microdomains, as it occurred in BAPTA-AM-treated cells. We applied the pharmacological SOC antagonist 2-APB (100 \(\mu\)M) to B16BL6-8 cells or transfected cells with shRNA against Stim1 and detected a 10-fold reduction of SOC entry rates (Figure 5A). This inhibition of SOC did not alter raft partition of calmodulin and Src (Figure 5B) but diminished their immune coprecipitation (Figure 5C) and significantly decreased expression of pAkt, pPP2Ac and pSrc (Figure 5D) that was consistent with our hypothesis. Importantly, application of thapsigargin alone did not change levels of these phosphoproteins (Supplementary Figure 1B is available at Carcinogenesis Online), indicating that the constitutively active Src/PP2A/PKB pathway does not undergo further hyperactivation by thapsigargin-induced \([\text{Ca}^{2+}]_{\text{cyt}}\)-rises but is sensitive to interruption of Ca\(^{2+}\) signal. Together with data presented in previous sections, these

Each blot represents one experiment of three performed. Aliquots of 50 \(\mu\)g (A–F) or 20 \(\mu\)g (G) of total protein extract were loaded per lane. Intensities of phosphoprotein expression were normalized to those of actin (mean + SD; \(n = 3\)). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\), n.s., not significant.

Fig. 3. Calmodulin and Src are coexpressed and Ca\(^{2+}\)-dependently bound in melanoma rafts. Total protein lysates prepared from control (A), M\(\beta\)CD-treated (14 mM, 2 h) (B), M\(\beta\)CD-treated (14 mM, 2 h) and cholesterol-replenished (50 \(\mu\)g/ml, 1 h) (C), W7 (50 \(\mu\)M, 3 h)- (D) and BAPTA-AM (50 \(\mu\)M, 3 h)-treated cells (E) were fractionated by ultracentrifugation in a sucrose gradient. Resulting fractions (1–9) were subjected to western blotting and dot blot analyses with indicated antibodies and horseradish peroxidase-conjugated CTXB, respectively. (F) Total protein lysates from M\(\beta\)CD-treated, M\(\beta\)CD-treated and cholesterol-replenished, W7- and BAPTA-treated cells were subjected to immune precipitation (IP) with calmodulin (CaM)-reactive antibodies. Immune complexes were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immune blotted (IB) to Src- and calmodulin-reactive antibodies. (G) Immune precipitation (IP) of Src with calmodulin-reactive antibodies from total protein lysates prepared from cells transiently transfected with pcDNA3-CaM\(_{1,2,3,4}\), pcDNA3-WT-CaM-encoding vectors and pcDNA3 control plasmids. (A–G) Each blot represents one experiment of three performed.
results suggest raft-mediated coupling of SOC via calmodulin to the downstream Src/PP2A/PKB cascade and thereby PKB activity.

Blockade of Src/PP2A/PKB pathway in the tumor site by treating melanoma-engrafted mice with raft-abating MβCD retards melanoma development and extends survival of tumor-bearing animals

We have next sought to determine the implication of this raft-dependent Src/PP2A/PKB pathway for in vivo melanoma growth and survival of melanoma-bearing mice. C57BL/J mice were inoculated into the footpad with B16BL6-8 cells and starting from day 8 after tumor cell implant were treated by intraperitoneal injections of raft-abating agent MβCD (300 mg/kg/week) (17). Tumor development and animal survival were monitored by measuring foot diameter until the endpoint of the experiment. All control (saline-treated) animals (n = 18) developed tumors. In MβCD-treated group (n = 18), tumors were detected in 83.33% (15/18) of animals and growth slowly, whereas pAkt, pPP2Ac and pSrc immune reactivity with more intense staining in perimembranous areas of malignant cells, whereas pPP2Ac staining was predominantly membranous (Figure 6D). In contrast, in MβCD-treated mice, tumors displayed barely detectable pAkt, pPP2Ac and pSrc immune reactivity, which were of significantly lower intensity compared with the control group (Figure 6D). Collectively, results presented in this section demonstrate the efficacy of in vivo raft disruption in abolishing deregulation of major tumor-promoting signaling elements, retarding tumor growth and prolonging animal survival that highlights the potential of raft-targeting compounds as effective anticancer drugs.

Discussion

The principal finding of the current study is that ablation of cholesterol-enriched lipid rafts interrupts a novel Ca2+-dependent mechanism of constitutive activation of PKB in malignant melanoma cells, attenuates tumor growth and greatly extends life span of melanoma-engrafted mice. Recently, we reported that in B16BL6 and additional melanoma lineages, intact raft and strong Ca2+ stimulus, both are critical determinants for enhanced PKB activity under basal conditions (17,18). We show now that abnormal PKB activation is coupled to these Ca2+ stimuli in the lipid rafts, where the binding of the Ca2+ sensor calmodulin to Src kinase promoted by Ca2+ influx via the SOC sustains Src activity and Src-induced inhibitory phosphorylation of PP2A, interfering with negative PKB regulation. Indication for this pathway was provided by the striking correlation between reduction in levels of active Src, stimulation of PP2A and inactivation of PKB.
triggered by alteration of lipid raft integrity with MβCD (Figure 1A) and inhibition of Ca²⁺/calmodulin (Figure 2A, D and G). The rescuing effect of the PP2A blockade on PKB but not Src activity altered by these manipulations (Figures 1C and 2B and E) suggested that, consistent with previous reports (25,36–38), PP2A acted downstream of Src and being repressed by this kinase at resting state, underwent reactivation following inhibition of Src. Indeed, direct blockade of Src was sufficient to reactivate PP2A and to abolish aberrantly high PKB activity (Figure 1E and F), whereas the reversal of PKB, PP2A and Src activities to their basal levels after lipid raft recovery in MβCD-treated cells (Figures 1B and 3A–C; Supplementary Figure 1A is available at Carcinogenesis Online) highlighted the major role of cholesterol-enriched microdomains in maintenance of entire Src/PP2A/PKB cascade. Our data further indicate that activation of Src and thereby downstream PP2A and PKB events required coexpression and Ca²⁺-dependent association of calmodulin with Src in the rafts that were aborted when interactions between these proteins disrupted by raft ablation or by interference with calmodulin function. Based on the following results (Figures 3–5), we concluded that calmodulin does not interact with Src directly but is, rather, permanently tethered to a putative raft-residing activator of Src and promotes binding of this activator to Src in the presence of Ca²⁺ stimulation. (i) The blocker of calmodulin substrate-binding pocket W7 (39) disrupted calmodulin/Src complexes by excluding calmodulin but not Src from the rafts. (ii) Elimination of Ca²⁺ signal with the intracellular Ca²⁺-chelator BAPTA-AM or by interrupting the store-operated Ca²⁺ influx abolished calmodulin/Src interactions but did not alter partition of these proteins in raft microdomains. Notably, raft integrity was preserved, indicating that approaches (i) and (ii) targeted Ca²⁺/calmodulin. Calmodulin did not activate PKB by binding this enzyme or its regulators PI-3K, PDK1 or focal adhesion kinase (18–21) and facilitating their recruitment to the rafts, as it may occur in growth factor- or cytoadhesion-stimulated cells. None of the latter proteins was detected in isolated rafts or pulled down with calmodulin-reactive antibodies (data not shown), arguing against this possibility. Finally, (iii) indirect interactions of calmodulin with Src were disrupted by overexpression of CaM1,2,3,4, consistent with the ability of this non-functional mutant to saturate Ca²⁺-insensitive tethering sites for its endogenous counterpart protein (32).

The proposed double-negative mechanism of constitutive PKB activation corresponds to a series of reports showing that the negative PKB modulator PP2A is repressed in many tumor types including melanoma (5,26,35,36,39–42). Mutational changes of the regulatory PP2A subunits (5,26) and overexpression of the endogenous phosphatase inhibitors (40,41) are widely recognized but, importantly, these alterations do not comprise a sole cause of PP2A dysfunction in

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**Fig. 5.** Inhibition of store-operated Ca²⁺ influx interferes with Ca²⁺-dependent stimulation of calmodulin and the downstream Src/PP2A/PKB cascade. (A) Effects of 2-APB (100 µM) and Stim1 shRNA on SOC activity were determined using Fluo-4-AM and flow cytometry. Timing of 2-APB addition is marked by red arrow. Left panel: Changes of mean of [Ca²⁺]ₘₑₙ ± SD in two independent experiments performed in triplicates. Right panel: Means ± SD of SOC entry rates are shown (n = 6). (B) Total protein lysates from control, 2-APB-treated (100 µM, 2 h) and Stim1 shRNA-expressing cells were fractionated by ultracentrifugation in a sucrose gradient and subjected to western blotting analysis of calmodulin (CaM) and Src expression. Partition of GM1 in cell fractions was assessed by dot blot analysis. (C) Immune precipitation of Src with CaM-reactive antibodies from total protein lysates prepared from control, 2-APB-treated (100 µM, 2 h) and Stim1 shRNA-transfected cells. (D) Total protein lysates (20 µg per lane) prepared from cells treated, as described in (C), were subjected to western blotting analysis of pAkt, pPP2Ac and pSrc expression. This analysis also confirmed downregulation of Stim1 levels in Stim1 shRNA-transfected cells. Intensities of phosphoprotein expression were normalized to those of actin (mean ± SD of two independent experiments). Each blot (B–D) represents one experiment of two performed. *P < 0.05, **P < 0.01, ***P < 0.001.
Fig. 6. Blockade of Src/PP2A/PKB pathway in the tumor site by treating melanoma-engrafted mice with MβCD retards melanoma development and extends survival of tumor-bearing animals. (A) Foot diameter was measured in mice engrafted with B16BL6-8 cells into the footpad and intraperitoneally treated by MβCD (300 mg/kg/week) (n = 18) or saline (control) (n = 18) during 56 days after cancer cell implant. Mean ± SD of two independent experiments are presented. (B) Survival of MβCD-treated versus control mouse in experiments described in (A) was monitored during 90 days after cancer cell implant. (C) MβCD-treated and control B16BL6-8 cell-engrafted mice were photographed 25 days after inoculation of tumor cells. Melanoma specimens (marked by black arrows) were obtained from these animals and subjected to immunohistochemistry with anti-pAkt, -pPP2Ac and -Src antibodies (D). (D) Upper panel: typical microphotographs of pAkt, pPP2Ac and pSrc immune reactivity in successive sections of melanoma tissues obtained from control and MβCD-treated mice (magnification ×20). Lower panel: immune staining intensities were determined as described in Materials and methods and expressed as mean ± SD; n = 5. *P < 0.05, **P < 0.01, ***P < 0.001.

cancer. Studies on leukemia (38), colon (36) and breast carcinoma cells (42) demonstrated that PP2A could as well be repressed through phosphorylation of the catalytic enzyme subunit by activated Src or other non-receptor and receptor tyrosine kinases. Furthermore, close association of Src activation and thereby Src-induced phosphorylation and inhibition of PP2A with intracellular Ca2+ rises demonstrated in neurons and other cell types (37,43) is in agreement with triggering of the Src/PP2A/PKB pathway by the Ca2+ sensor calmodulin in malignant melanoma cells, in which accelerated function of SOC maintains moderately elevated steady state [Ca2+]i (17,18). SOC is predominant Ca2+ entry route in non-excitable cells, stimulated by many membrane receptors and mediated by depletion of intracellular Ca2+ stores (44). Lipid rafts have been reported to modulate SOC by creating a platform for interactions of different SOC-associated proteins (27–29). Particularly Stim1 targeted to the rafts might assemble with TRPC and/or Orai constituents of Ca2+-permeation pore of the SOC and elicit Ca2+ entry. Growing evidence now implicates robust SOC responses in enhanced proliferation, survival and invasion of melanoma (17,18), breast (45,46), colon (47,48), lung (49,50) and hepatocellular carcinoma cells (51). However, the pathways linking these channels to sustained deregulation of major tumor-promoting signaling elements are not fully understood. Thus, the recruitment of Stim1 to melanoma rafts, triggering Ca2+ influx and Ca2+/calmodulin-dependently stimulating the Src/PP2A/PKB cascade (Figures 4 and 5), demonstrates for the first time that such link is mediated by cholesterol-enriched microdomains connecting SOC-induced [Ca2+]i rises to high basal PKB activity, which is critical for melanoma malignancy. At present we do not know the identity of raft-associated Src activator/s, involved in coupling of calmodulin to Src. It yet remains to be determined whether the latter activator/s are adaptors proteins, such as arrestins (52) or tyrosine kinase- and G-coupled receptors (53,54), bound and Ca2+-dependently modulated by calmodulin. Notably, stimulation of calmodulin by the SOC also evokes activation of nuclear factor of activated T cells and nuclear factor-kappaB transcription factors (55,56). Recent studies on melanoma (57), colon (48) and lung carcinoma cells (50) indicated a role of these proteins in transcriptional upregulation of metastasis-related genes. Since PKB promotes nuclear translocation and thereby activities of both transcription factors (58,59), it would be of particular interest to examine potential crosstalk between PKB and nuclear factor-kappaB SOC-driven signaling and its significance for cancer invasion. Regardless of these unresolved issues, the obvious dependence of the Src/PP2A/PKB cascade on lipid raft integrity by itself implies important therapeutic implication, as targeting rafts in vivo might restrain melanoma development by reactivating PP2A and inhibiting PKB in the tumor site.

As such, we have used MβCD, a cyclic oligosaccharide with capability to eliminate rafts by mobilizing membrane cholesterol from cultured (30) and tissue-residing cells (60), for in vivo raft disruption and assessed activation status of the Src/PP2A/PKB pathway in tumors derived from MβCD-treated mice. A major concern to this paradigm is that MβCD or related β-cyclodextrins might non-specifically interfere with raft function in vital body organs and trigger severe systemic toxicity. Completely opposite to this notion, low
Fig. 7. Proposed double-negative mechanism of raft-dependent constitutive PKB activation via SOC, Ca\(^{2+}\)/calmodulin, Src and PP2A. (A) Lipid rafts generate ‘platforms’, at which Stim1 triggers SOC-mediated Ca\(^{2+}\) influx (1) and promotes Ca\(^{2+}\)-driven indirect interactions of raft-residing calmodulin (CaM) with Src, sustaining Src activity (2) and Src-induced inhibition of PP2A (3). This compromises negative regulation of PKB by PP2A and preserves PKB in permanently active state (4). Calmodulin is Ca\(^{2+}\)-independently tethered to the putative raft-residing Src activator (black bar) and facilitates binding of this activator to Src in the presence of Ca\(^{2+}\) stimulation (red circles). (B) Elimination of raft platforms in M\(\beta\)CD-treated cells interferes with Stim1-mediated triggering of the SOC (5), disrupts calmodulin–Src complexes (6), reactivates PP2A by inhibiting Src (7) and abolishes high basal PKB activity (8).

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Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/.

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