Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 336 (2005) 3–8
BBRC
www.elsevier.com/locate/ybbrc

Transcriptional signaling from membrane raft-associated glucocorticoid receptor

Sudhir Jain [a], Yanna Li [a], Ashok Kumar [a], Pravin B. Sehgal [b,c,*]

[a] Department of Pathology, New York Medical College, Valhalla, NY 10595, USA
[b] Department of Cell Biology and Anatomy, New York Medical College, Valhalla, NY 10595, USA
[c] Department of Medicine, New York Medical College, Valhalla, NY 10595, USA

Received 5 August 2005
Available online 18 August 2005

Abstract

The contribution of plasma membrane-associated glucocorticoid receptor (GR) to transcriptional signaling is unclear. We observed GR in low-density detergent-resistant membrane (DRM) rafts derived from human hepatoma Hep3B cells in complexes with caveolin-1, HSP90, and STAT3. In transient transfection assays, GR-stimulated transcriptional signaling was reversibly inhibited by membrane-raft disrupters filipin III and progesterone. These data provide clear evidence for a functional contribution of DRM-associated GR to transcriptional signaling.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Glucocorticoid receptor; Membrane rafts; IL-6; STAT3; Caveolin-1; Transcriptional signaling

The existence of pools of plasma membrane-associated receptors for respective steroid hormones such as estrogens, glucocorticoids, and aldosterone is clearly established [1–7]. The functional role of these membrane-associated steroid receptors is now under intensive study. Both genomic and non-genomic effects have been attributed to the activation of such membrane-associated steroid receptors [1–7]. As an example, plasma membrane-associated ER, which is localized to complexes with caveolin-1, contributes towards transcriptional signaling (a "genomic" effect) [1] as well as towards the functional modulation of other raft-associated enzymes such as eNOS (a "non-genomic" effect) [8].

While pools of plasma membrane-associated glucocorticoid receptor (GR) have been clearly established (e.g., in the plasma membrane of the hepatocyte) [3,4], and sometimes referred to as membrane-GR or mGR [3,9], the function of this pool of GR remains controversial. For instance, does this pool contribute towards transcriptional signaling? In this communication, we address this question. We provide evidence for (a) localization of GR to detergent-resistant low-density membrane rafts (DRMs) in hepatocytes and (b) for the contribution of DRM-localized GR towards functional transcriptional signaling.

Materials and methods

Cell culture. Human hepatoma cells (Hep3B) were grown as monolayers in Dulbecco's modified Eagle's medium as described [10]. Cells were grown to confluence in 100 mm plastic Petri dishes for membrane-raft preparation and to partial confluence in 6-well plates for transfection assays. Prior to the indicated experimental treatments, these cultures were washed twice with PBS and replenished with serum-free medium. Various experimental treatments included dexamethasone (100 nM; Sigma–Aldrich D4902) for 3 h, IL-6 (10 ng/ml; R&D Systems) for 30 min, filipin III (10 ng/ml; Sigma–Aldrich F4767) for 30 min (prior to the experiment), progesterone (10 μg/ml; Sigma–Aldrich P6149) for 5 h alone or in combinations as indicated in respective figure legends.

* Corresponding author. Fax: +1 914 594 4825.
E-mail address: pravin_sehgal@nymc.edu (P.B. Sehgal).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.057

Cell fractionation and preparation of DRMs. Hep3B cell cultures in 100 mm plates were harvested by scraping into ice-cold phosphate-buffered saline (PBS), washed twice with ice-cold PBS, and resuspended in ~0.8 ml of hypotonic extract lysis buffer (ELB) [11–13]. As described earlier [11–13], the cells were broken by Dounce homogenization and fractionated further by differential centrifugation into a nuclear pellet and a post-nuclear cytoplasm (PNS). The PNS was further divided into a 15,000*g* (15 min) membrane pellet and a supernatant. The P15 membrane pellet was washed once with 0.5 ml ELB, resedimented, and then resuspended in 50 μl ELB. As previously characterized, the P15 pellet consists of plasma membrane sheets together with large cytoplasmic granular elements [11,12] and provides a cytosol-depleted starting material for preparing plasma membrane rafts [11,12]. Detergent-resistant membrane rafts (DRMs) were derived from the P15 fraction using the Triton X-100 flotation procedure as described by Lafont et al. [14] as modified by us [11,12]. Eight or 12 equal fractions were collected from each gradient from the top and used for further analyses. The specific detergent-resistant low-density caveolin-1-positive raft fractions near the top of such flotation gradients have been extensively characterized by us earlier to include the co-flotation of additional markers such as the flotillins as well as the gp130 signal transducing chain of the IL-6 receptor [11,12].

Western blot analysis. Western blotting was carried out using 12.5% SDS–polyacrylamide gels under reducing denaturing conditions in accordance with procedures and protocols provided by Bio-Rad Laboratories (Hercules, CA, USA), and ECL detection kit (Amersham Biosciences) [11–13]. Monoclonal antibodies (mAbs) to glucocorticoid receptor (GRs), STAT3, HSP90, and caveolin-1 and polyclonal antibody to GR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Magnetic-bead immunopanning. This process was carried out using rabbit anti-GR IgG essentially as described earlier [11,12]. Protein A magnetic beads were purchased from New England Biolabs (Beverly, MA). Prior to use in immunopanning (IPN) experiments, the beads were blocked with 5% non-fat dried milk in PBS for 1 h and then washed three times with PBS. Aliquots of relevant membrane flotation fractions were adjusted to 0.05% Triton X-100 in 25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1 mM dithiothreitol (“binding buffer”), and incubated overnight at 4 °C with respective rabbit immunoglobulin (non-immune rabbit serum or anti-GR pAb) and then for 1 h at 4 °C with the pre-blocked protein A magnetic beads. Immunopanned protein complexes were analyzed by SDS–PAGE and Western blotting under reducing denaturing conditions [11–13]. The specificity of complexes isolated using magnetic-bead immunopanning assays, particularly using the Triton X-100 (0.5%)/SDS (0.1%) washing buffer, has been extensively documented by us earlier using a variety of non-immune IgG as well as in assays without inclusion of any antibody [11,12].

Reporter/luciferase plasmid constructions. The synthesis of reporter construct pHAGT1.3 *luc* (containing 1.3 kb of human AGT gene promoter attached to the luciferase gene) and $(217A)_6$-*luc* (containing a GR-binding site) has been described earlier [10]. Reporter construct $(950M)_4$-*luc* (containing STAT3-binding site) was constructed by tetramerization of oligonucleotide CGTTTCTGGGAACCT and blunt-ended ligation of the multimer in the *Sma*I site of pGL3 promoter [10,11,15].

Transient transfection assays. Transfections in Hep3B cell cultures in 6-well plates were carried out using the LipofectAMINE reagent (Polyfect, Qiagen, Valencia, CA) and the manufacturer’s protocol [10,11,15]. All experiments were conducted in triplicate in at least three independent transfections. For co-transfection experiments, 50 ng of constitutive expression vectors for GR (RSV-GR plasmid), STAT3 (pSTAT3) was added to respective reporter constructs (250 ng) and the final amount of transfected DNA per well was adjusted to 1 μg by the addition of Bluescript DNA. After 4 h of incubation with the transfection mixture, the culture medium was changed. Twenty-four hours later, the cultures were treated as indicated in the respective figure legends. The reversal of progesterone and filipin effects was achieved by washing the cells twice with PBS, followed by addition of regular media. Whole cell extracts were prepared by resuspension in 200 μl lysis buffer (Promega, Madison, USA). Luciferase activity was normalized for the β-gal activity, which was determined as described previously [10,15].

Statistical analysis. The unpaired two-tailed *t* test was used to compare the relative luciferase activities of reporter constructs in transient transfection experiments.

Results

GR in detergent-resistant caveolin-1-containing low-density membrane rafts

Triton X-100-resistant low-density rafts were prepared by flotation of the cytosol-depleted P15 membrane derived from Hep3B cells and the respective fractions were assayed for GR, cav-1, and the transcription factor STAT3 by Western blotting. Fig. 1A shows the presence of GR in membrane-associated flotation fractions all the way to the lightest fractions, including the fraction enriched in caveolin-1 (cav-1). Consistent with our previous data [11,12], Fig. 1A also shows the inclusion of the transcription factor STAT3 in such membrane fractions, especially the low-density raft fraction (#2 in Fig. 1A). Fig. 1B shows that the addition of Dex to Hep3B cells from which the P15 fraction was prepared increased the amount of GR that was associated with DRMs (compare panels a and b in Fig. 1B). Physically, this membrane-associated GR was present in detergent-resistant complexes together with HSP90, STAT3, and caveolin-1, as assayed in immunopanning experiments (Fig. 1C).

Filipin III inhibits Dex/GR function

In previous studies [11,12], we have used the raft disrupter filipin III in order to evaluate the contribution of raft components to functional signaling. For example, we have previously shown that filipin III inhibits IL-6-induced STAT3 signaling in order to assess the functional role of STAT3 in rafts. In a similar manner, we evaluated whether GR present in rafts might functionally contribute to transcriptional signaling using filipin III. For this purpose, we cloned a 1.3 kb of human angiotensinogen gene promoter to make 1.3hAGT (which contains glucocorticoid and IL-6-responsive elements), and also multimers of glucocorticoid (217A6) and IL-6 (950M4) responsive elements from AGT gene promoter in luciferase reporter vector. Fig. 2 is a composite representation of several transient transfection experiments using the above three reporter constructs [11,12]. As a positive control, Fig. 2A shows that filipin III markedly but reversibly inhibited IL-6-induced activation of the STAT3-responsive reporter construct 950M4. Figs.

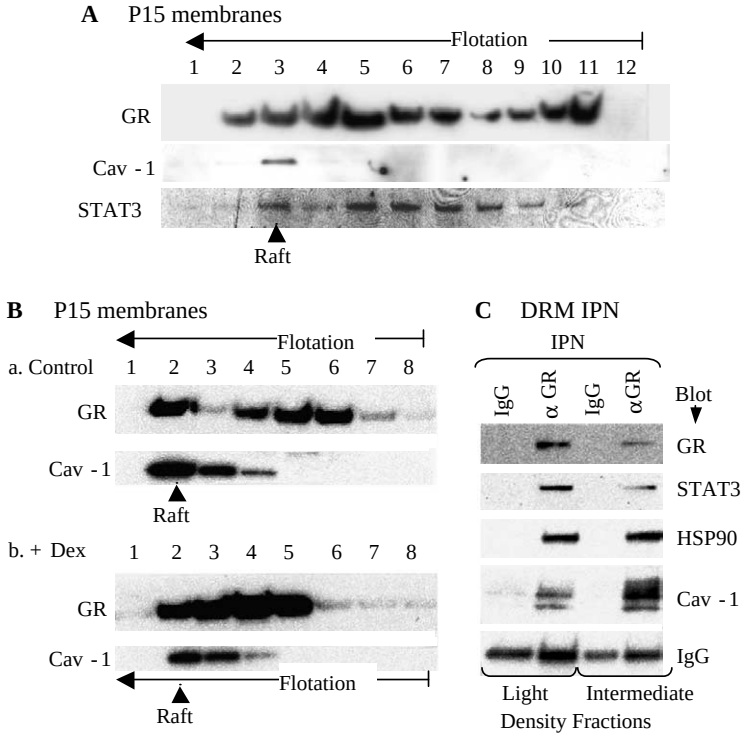

Fig. 1. GR in DRMs and GR complex with cav-1, STAT3, and HSP90 in Hep3B hepatocytes. P15 membrane fractions derived from untreated (A,Ba) or Dex-treated (Bb) Hep3B cells were subjected to equilibrium flotation as described in Materials and methods. Western blots were carried out using 50 μl of each flotation fraction. (C) Immunopanning was carried out from pooled flotation fractions #3, 4 and #5, 6 from (Ba) using anti-GR antibody and non-immune IgG as control and the immunopanned protein complexes were analyzed by SDS–PAGE under reducing denaturing conditions and Western blotting. The blot was probed sequentially with cav-1, STAT3, and HSP90 mAbs.

2B–D confirm that filipin III inhibited Dex/GR-stimulated activation of the respective reporter constructs, and that this inhibition was not due to cellular toxicity in that the inhibition was reversible. These experiments indicate a functional role of GR in plasma membrane DRMs towards transcriptional activation.

Effect of a second raft disrupter on GR function

Anderson and co-workers [16] have pioneered the use of progesterone as a raft disrupter based upon its ability to inhibit cholesterol and caveolin-1 trafficking to and from the plasma membrane. A consequence of progesterone treatment is the disruption of cholesterol rafts. Thus, in addition to filipin III, we used progesterone to evaluate the functional importance of rafts in Dex-induced GR transcriptional signaling.

The transcriptional assay data in Fig. 3 show the ability of progesterone to reversibly inhibit Dex/IL-6/STAT3 signaling. As with filipin III, as a positive control, progesterone markedly but reversibly inhibited IL-6/raft signaling (Fig. 3A). This raft-disruptive treatment clearly inhibited Dex-induced transcriptional alone (Figs. 3B and C) or in addition to IL-6 or with the further transfection of a STAT3 expression vector (Fig. 3D). Importantly, Fig. 4A confirms that progesterone markedly inhibited the presence of cav-1 in low-density detergent-resistant membrane raft fractions per se. Fig. 4B shows that under similar conditions there is also a marked inhibition of the localization of GR in the DRM fractions. Taken together, these data provide further evidence for a functional role of GR in plasma membrane DRMs in transcriptional activation.

Discussion

The present data provide clear evidence for the functional contribution of membrane raft integrity in the transcriptional activation program stimulated by dexamethasone. The constitutive localization of GR in DRMs and the ability of Dex to further recruit GR to DRMs are consistent with these functional data. Moreover, the inclusion of GR in DRMs in physical complexes with caveolin-1, HSP90, and STAT3 provides a novel basis for the regulation of steroid function by these molecules at the level of the plasma membrane raft.

The data in Figs. 2A and 3A showing the ability of filipin III and progesterone to markedly but *reversibly* inhibit *transcriptional* activation by IL-6 of a STAT3-responsive reporter (p950M4) provide important new evidence in support of the IL-6/raft/STAT3 signaling hypothesis. Previously, evidence for a functional contribution of cholesterol-rich rafts in cytokine/STAT signal-

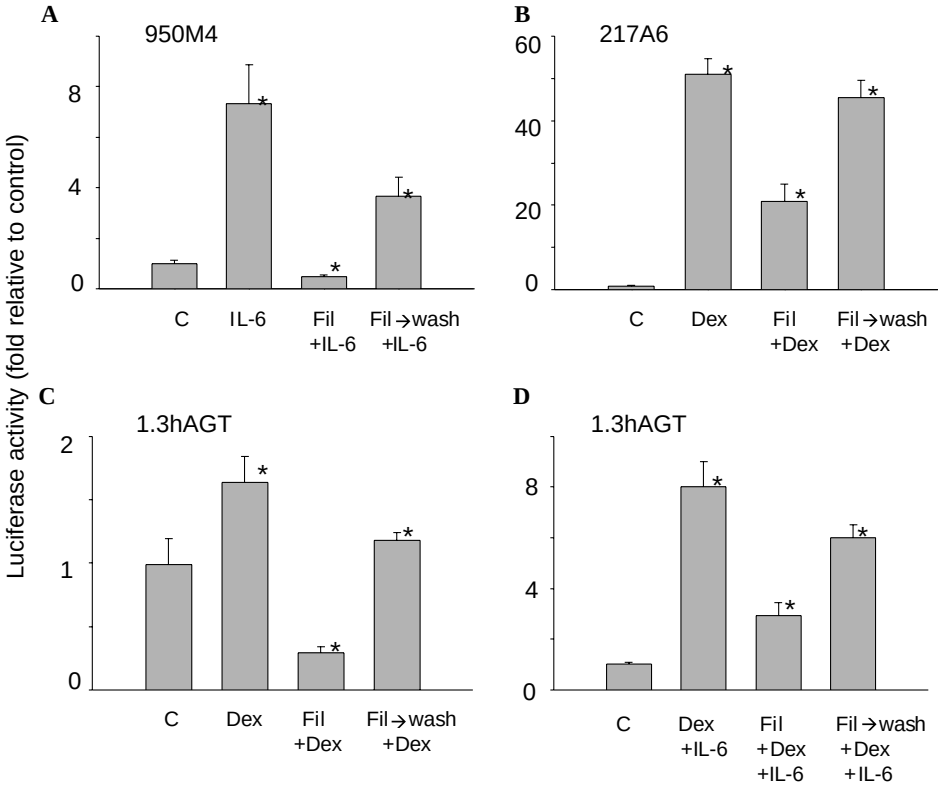

Fig. 2. Effect of the raft disrupter filipin III on GR/Dex/IL-6-induced promoter activity of luciferase reporter constructs. Hep3B cells were transiently transfected with three different luciferase reporter constructs generated from human angiotensinogen promoter. Twenty-four hours later, the cultures were exposed to Dex (for 6 h) or IL-6 (for 6 h). To assay for the effect of filipin III, this inhibitor was added 30 min prior to the addition of Dex or IL-6 (alone or in combination). To assay for reversibility, the filipin III was washed out with cold PBS just prior to the addition of Dex or IL-6. (A) Luciferase activity using 950M4 construct (IL-6/STAT3-responsive element); (B) luciferase activity using 217A6 construct (glucocorticoid-responsive element); and (C and D) luciferase activity using 1.3 kb hAGT promoter construct (containing both glucocorticoid and IL-6/STAT3-responsive elements). Luciferase activity (means ± SE; $n \geqslant 3$; normalized with respect to β-galactosidase as a transfection control) is expressed in terms of that observed in untreated cultures. Asterisks denote $P < 0.05$.

ing has come from the use of filipin III or β-methyl cyclodextrin as raft disrupters in short-term experiments lasting, for example, for 60 min [11,12]. In these experiments, the assays for STAT signaling have been either ligand-induced STAT-specific Tyr-phosphorylation or acquisition of DNA-binding activity. Thus, up to now there was no evidence that rafts contributed to the transcriptional activation function per se of the cytokine/STAT signaling cascade. Moreover, these previous experiments were open to the critique that, under the experimental conditions used, there might have occurred a loss of plasma membrane integrity [17]. Thus, it was necessary to investigate the effect of raft disrupters under conditions that: (a) did not produce a leakage of cytosolic lactate dehydrogenase, (b) carry out assays for transcriptional activation per se, and (c) show that washing out the inhibitors restored transcriptional signaling. As previously reported by Anderson and coworkers [16], we confirmed the ability of progesterone to inhibit cav-1 localization to DRMs, as well as alterations in DRM-associated cav-1 following filipin III treatment (data not shown). Under such experimental conditions, the transient transfection data in Figs. 2A and 3A showing the ability of the raft disrupters filipin III and progesterone to reversibly inhibit transcriptional activation by IL-6 provide new functional evidence in support of the IL-6/raft/STAT3 signaling hypothesis.

The constitutive presence of GR in DRMs and the Dex-induced further recruitment of GR to DRMs (in Fig. 1B compare control gradients vs. +Dex) raised the question about the contribution of this membrane-associated pool to GR to the transcriptional activation function of glucocorticoid hormone. The transient transfection data in Figs. 2 and 3 address this question and show that the raft disrupters filipin III and progesterone both inhibited Dex-induced activation of two different GRE-containing reporter constructs in a reversible manner. The association of GR with cav-1, HSP90, and STAT3 in detergent-resistant complexes (Fig. 1C) places some of the molecules known to modulate GR and be affected by GR in the membrane raft. The specific contribution of each of these modulatory

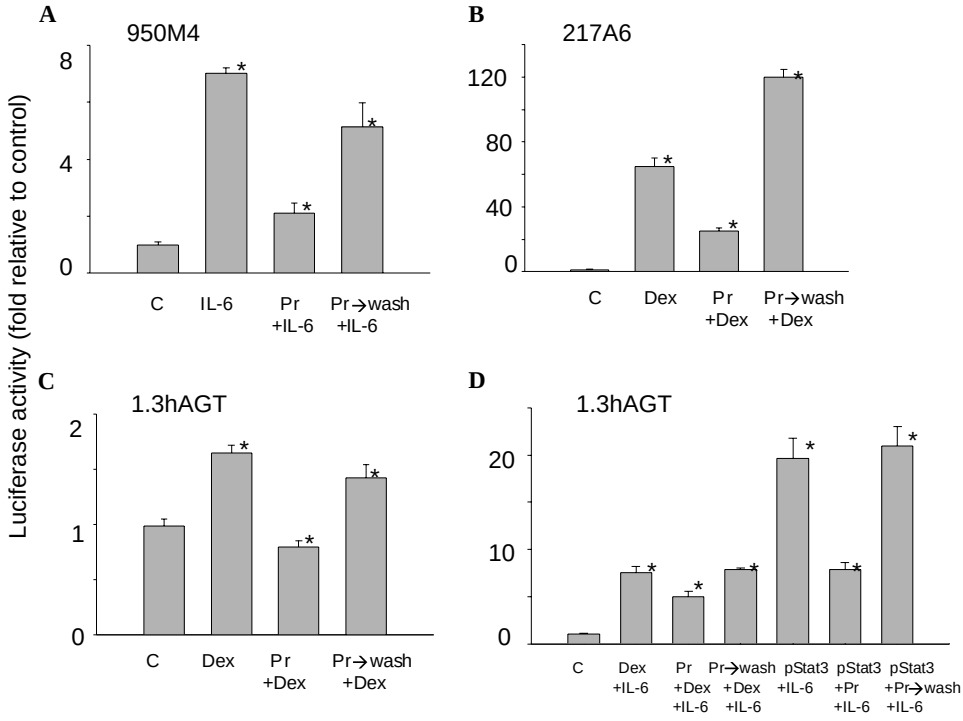

Fig. 3. Effect of the raft disruptor progesterone on GR/Dex/IL-6-induced promoter activity of luciferase reporter constructs. Hep3B cells were transiently transfected with different luciferase reporter constructs and after 24 h of transfection were treated with Dex (for 6 h) or IL-6 (for 6 h). To assay for the effect of progesterone, this inhibitor was added 1 h prior to the addition of Dex or IL-6 (alone or in combination). To assay for reversibility, the progesterone was washed out with cold PBS just prior to the addition of Dex or IL-6. (A) Luciferase activity using 950M4 construct; (B) luciferase activity using 217A6 construct; and (C and D) luciferase activity using 1.3 kb of hAGT promoter construct. In separate experiments, we have confirmed that there is no loss of lactate dehydrogenase from cell extracts upon treatment with progesterone (data not shown). Luciferase activity (means ± SE; $n \geqslant 3$; normalized with respect to β-galactosidase as a transfection control) is expressed in terms of that observed in untreated cultures. Asterisks denote $P < 0.05$.

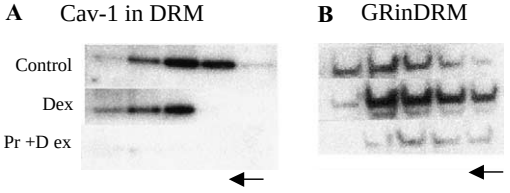

Fig. 4. Progesterone inhibits localization of cav-1 and GR in DRM fractions. (A) Hep3B cells were treated with Dex in the presence or absence of Pr (total time 6 h) and the DRM fractions were prepared from the isolated P15 membrane fractions as indicated in the legend to Fig. 1. The panel illustrates Western blotting data for cav-1 from fractions in the low-density DRM region. (B) Hep3B cells were treated with IL-6 and Dex in the presence or absence of Pr for 6 h. DRM fractions were prepared from the isolated P15 membrane fractions as indicated in the legend to Fig. 1. The panel illustrates Western blotting data for GR from fractions in the low-density DRM region. Arrows indicate direction of flotation.

molecules to raft/GR transcriptional signaling as well as the downstream pathway from the membrane raft to the nucleus (such as via a "signaling endosome"; [18]) remains to be evaluated.

To summarize, the present data show the presence of GR in low-density cav-1-containing DRMs derived from liver cells and provide evidence for the functional contribution of GR in rafts towards transcriptional signaling.

Acknowledgments

This work was supported in part by Research Grants RO1 HL49884 and RO1-HL 59547(to A.K.) and R01-HL-077301 (to P.B.S.) from The National Institutes of Health, USA, and a grant from Philip Morris Inc. (to A.K.).

References

[1] A. Schlegel, C. Wang, B.S. Katzenellenbogen, R.G. Pestell, M.P. Lisanti, Caveolin-1 potentiates estrogen receptor alpha (ERalpha) signaling. Caveolin-1 drives ligand-independent nuclear translocation and activation of ERalpha, J. Biol. Chem. 274 (1999) 33551–33556.
[2] M. Wehling, Specific, nongenomic actions of steroid hormones, Annu. Rev. Physiol. 59 (1997) 365–393.
[3] R.M. Losel, E. Falkenstein, M. Feuring, A. Schultz, H.C. Tillmann, et al., Nongenomic steroid action: controversies, questions, and answers, Physiol. Rev. 83 (2003) 965–1016.

[4] S. Daufeldt, R. Lanz, A. Allera, Membrane-initiated steroid signaling (MISS): genomic steroid action starts at the plasma membrane, J. Steroid Biochem. Mol. Biol. 85 (2003) 9–23.
[5] B. Boldyreff, M. Wehling, Rapid aldosterone actions: from the membrane to signaling cascades to gene transcription and physiological effects, J. Steroid Biochem. Mol. Biol. 85 (2003) 375–381.
[6] T. Simoncini, A.R. Genazzani, Non-genomic actions of sex steroid hormones, Eur. J. Endocrinol. 148 (2003) 281–292.
[7] C.S. Watson, B. Gametchu, Proteins of multiple classes may participate in nongenomic steroid actions, Exp. Biol. Med. (Maywood.) 228 (2003) 1272–1281.
[8] T. Caulin-Glaser, G. Garcia-Cardena, P. Sarrel, et al., 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca^{2+} mobilization, Circ. Res. 81 (1997) 885–892.
[9] C.E. Powell, C.S. Watson, B. Gametchu, Immunoaffinity isolation of native membrane glucocorticoid receptor from S-49++ lymphoma cells: biochemical characterization and interaction with Hsp 70 and Hsp 90, Endocrine 10 (1999) 271–280.
[10] S. Jain, X. Tang, C.S. Narayanan, et al., Angiotensinogen gene polymorphism at-217 affects basal promoter activity and is associated with hypertension in African-Americans, J. Biol. Chem. 277 (2002) 36889–36896.
[11] M. Shah, K. Patel, V.A. Fried, P.B. Sehgal, Interactions of STAT3 with caveolin-1 and heat shock protein 90 in plasma membrane raft and cytosolic complexes. Preservation of cytokine signaling during fever, J. Biol. Chem. 277 (2002) 45662–45669.
[12] P.B. Sehgal, G.G. Guo, M. Shah, V. Kumar, K. Patel, Cytokine signaling: STATS in plasma membrane rafts, J. Biol. Chem. 277 (2002) 12067–12074.
[13] M.I. Ndubuisi, G.G. Guo, et al., Cellular physiology of STAT3: where's the cytoplasmic monomer? J. Biol. Chem. 274 (1999) 25499–25509.
[14] F. Lafont, P. Verkade, T. Galli, et al., Raft association of SNAP receptors acting in apical trafficking in Madin-Darby canine kidney cells, Proc. Natl. Acad. Sci. USA 96 (1999) 3734–3738.
[15] S. Jain, Y. Li, S. Patil, A. Kumar, A single-nucleotide polymorphism in human angiotensinogen gene is associated with essential hypertension and affects glucocorticoid induced promoter activity, J. Mol. Med. 83 (2005) 121–131.
[16] E.J. Smart, Y. Ying, W.C. Donzell, R.G. Anderson, A role for caveolin in transport of cholesterol from endoplasmic reticulum to plasma membrane, J. Biol. Chem. 271 (1996) 29427–29435.
[17] A. Balbis, G. Baquiran, C. Mounier, B.I. Posner, Effect of insulin on caveolin-enriched membrane domains in rat liver, J. Biol. Chem. 279 (2004) 39348–39357.
[18] C.L. Howe, J.S. Valletta, A.S. Rusnak, W.C. Mobley, NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway, Neuron 32 (2001) 801–814.