Cytokine Signaling

STATS IN PLASMA MEMBRANE RAFTS*

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Abstract

STAT transcription factors signal from the plasma membrane to the nucleus in response to growth factors and cytokines. We have investigated whether plasma membrane “rafts” are involved in cytokine-activated STAT signaling. Cytokine-free human hepatoma Hep3B cells or cells treated with interleukin-6 (IL-6) or orthovancanade (a general activator of STATs) were fractionated, and plasma membrane raft fractions were obtained by equilibrium sedimentation or flotation through discontinuous sucrose gradients using either non-detergent or detergent-based (saponin or Triton X-100) methods. By Western blotting the plasma membrane raft fractions obtained using either non-detergent or detergent-based methods contained significant amounts of STAT1 and STAT3 (up to ~10% of the total cytoplasmic amount) as well as the integral raft proteins caveolin-1 and flotillin-1, the IL-6-receptor signal transducing chain gp130, the interferon-γ receptor α chain (IFN-γRα), and the chaperone glucose-regulated protein 58 (GRP58/ER-60/ERp57). Upon activation of signaling by IL-6 or orthovanadate the respective Tyr-phosphorylated STAT species were now also observed in the membrane raft fraction but in a form deficient in DNA binding. The data show pre-association of STATs with plasma membrane rafts in flotation fractions, which also contained caveolin-1 and flotillin-1, and suggest that Tyr phosphorylation may not in itself be sufficient to cause the departure of PY-STATs from plasma membrane rafts. Methyl-β-cyclodextrin, which sequesters cholesterol and disrupts plasma membrane rafts, markedly inhibited IL-6- and IFN-γ-induced STAT signaling. Signaling through specialized raft microdomains may be a general mechanism operating at the level of the plasma membrane through which cytokines and growth factors activate STAT species (the “raft-STAT signaling hypothesis”).

Signal transduction in mammalian cells is initiated by complex protein-protein interactions between ligands, receptors, and kinases at the level of the plasma membrane. It is now becoming clear that specialized microdomains at the cell surface, known as rafts and/or caveolae, are intimately involved in this process (1, 2). These lipid microdomains contain high concentrations of glycolipids, sphingomyelin, and cholesterol and represent platforms for conducting cellular functions such as vesicular trafficking and signal transduction (1, 2). Raft domains contain several integral raft proteins, which include caveolin and flotillin family members. Signaling processes shown to involve plasma membrane raft domains include immunoglobulin E signaling, T-cell antigen receptor signaling, B-cell receptor signaling, signaling involving epidermal growth factor, platelet-derived growth factor, insulin receptor, Ephrin B1 receptor, neurotrophin, Ha-Ras, nitric-oxide synthase and integrins (reviewed in Refs. 1–4). Caveolin-1 in rafts has been shown to modulate insulin- and Ha-Ras-mediated signaling (the “caveola signaling hypothesis”) (3, 4). Despite these major advances in the understanding of rafts as a unit of function of the plasma membrane, the role of plasma membrane rafts in cytokine-induced signaling remains largely unexplored.

A feature of many cytokines is that they engage the cell at the level of the non-tyrosine kinase cell surface receptors, which signal to the cell nucleus by activation of the JAK-STAT1 (Janus kinase signal transducer and activator of transcription) signaling pathway (5–8). As examples, interleukin-6 (IL-6) predominantly activates STAT1, and to a lesser extent STAT3, whereas interferon-γ (IFN-γ) predominantly activates STAT1 (5–8). In the standard model of STAT signaling (5–8), upon cytokine-induced receptor activation, monomeric cytosolic STATs are thought to be recruited to the cytoplasmic tail of the respective plasma membrane receptor, Tyr-phosphorylated by JAK family kinases, then they depart the receptor by an unknown mechanism, dimerize, and translocate to the nucleus. In the nuclear compartment PY-STATs bind target DNA motifs and other transcription factors and thus modulate gene expression.

In contrast to the standard model of STAT signaling, we and others (9–12) previously reported that STAT proteins were present in the cytosol of mammalian cells not as free monomers but in the form of high molecular mass complexes of size 200–400 kDa and 1–2 MDa. In our cell fractionation studies of human hepatoma Hep3B cells we noticed that ~5–10% of total cellular STAT3 and significant amounts of PY-STAT3 were

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Dedicated to the memory of Dr. Andreas Scheid, a valued teacher and colleague, and an expert on the biology of viral glycoproteins in the plasma membrane of enveloped viruses.

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1 The abbreviations used are: JAK, Janus kinase; STAT, signal transducer and activator of transcription; 5′-ND, 5′-nucleotidase; GRP78, glucose-regulated protein 78; BP, binding protein for immunoglobulin; ELB, extract lysis buffer; GRP58, glucose regulated protein GRP58/ER-60/ERp57; IFN, interferon; IL, interleukin; MCD, methyl-β-cyclodextrin; Nup62, nuclear pore-associated protein 62; PBS, phosphate-buffered saline; TRAP1, tumor necrosis factor receptor-associated protein 1; VLA-2α, very late activation antigen-2α; LDH, lactate dehydrogenase; pAb, polyclonal antibody; NE, nuclear extract; PY-STAT, Tyr-phosphorylated STAT; SIF-A, -B, -C, the A, B, and C complexes binding to the m67 mutant of the serum-inducible element from the c-fos promoter.
associated with a cytoplasmic membrane fraction (see Fig. 2 of Ref. 9, fraction designated MP). We have now determined that STATs associated with cytoplasmic membranes represent STATs contained in plasma membrane raft microdomains. Furthermore, the compound methyl-β-cyclodextrin (MCD), which is known to disrupt plasma membrane raft function, markedly inhibited IL-6- and IFN-γ-induced STAT signaling.

MATERIALS AND METHODS

Cell Lines

The parental human hepatoma Hep3B cell line and a derivative cell line ("Line 1") have been described earlier (9, 13). Line 1 Hep3B cells were used in the experiments reported here.

Cytokine and Orthovanadate Treatment

Hep3B cells were grown to confluence in 100-mm Petri dishes at 37 °C as described earlier (9, 13). For IL-6 or IFN-γ treatment, cultures were washed twice with phosphate-buffered saline (PBS), replenished with 5 ml of serum-free medium for 4 h, and then exposed to the reconstituent cytokine (10 ng/ml, R & D Systems Inc., Minneapolis, MN) for 15 or 30 min. We have also used orthovanadate alone as a general activator of STAT signaling in our experiments (5–8); for orthovanadate treatment, cultures in serum-free medium were treated with orthovanadate of 0.5 mM (orthovana-0.05% Triton X-100 flotation procedure essentially as described by Lafont and Si-dered fraction 1 located just above the 30/40% interface, fraction 2 (the 0/25% sucrose interface), fraction 3, the 43/60% sucrose interface; this was collected, diluted in ELB, and resedi-

Cell Fractionation

Cells were harvested by scraping into ice-cold PBS, washed twice with ice-cold PBS, resuspended in −0.5 ml extract lysis buffer (ELB, 10 mM Hepes, pH 7.9, 10 mM NaCl, 3 mM MgCl2, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, and 0.1 mM sodium vanadate) per cell pellet derived from five 100-mm culture, and fractionated into cytoplasmic and nuclear fractions by gentle cell breakage in a loose fitting Dounce homogenizer as described earlier (9, 13). All cell fractiona-
tion and gradient centrifugation steps were carried out at 4 °C. Nuclear pellets were removed from the cell homogenate by low speed centrifugation (1000 rpm for 3 min) for our experiments (5–8); for orthovanadate treatment, cultures in serum-free medium were treated with orthovanadate of 0.5 mM (orthovana-
to adjust the 70 ml sucrose, 220 mM mannitol, and 2 mM Hepes, pH 7.4 ("isolation medium") (2.8 ml for extract derived from fifteen 100-mm cultures). This cytoplasmic extract was then subjected to cen-
refraction and isolation medium and 60% sucrose (design-

Cytokine and Orthovanadate Treatment

Hep3B cells were grown to confluence in 100-mm Petri dishes at 37 °C as described earlier (9, 13). For IL-6 or IFN-γ treatment, cultures were washed twice with phosphate-buffered saline (PBS), replenished with 5 ml of serum-free medium for 4 h, and then exposed to the reconstituent cytokine (10 ng/ml, R & D Systems Inc., Minneapolis, MN) for 15 or 30 min. We have also used orthovanadate alone as a general activator of STAT signaling in our experiments (5–8); for orthovanadate treatment, cultures in serum-free medium were treated with orthovanadate of 0.5 mM (orthovanadate solution diluted with 40 volumes of ELB and resedimented, and then resuspended in ELB (50- to 100-fold dilution). All except fraction 6 (which represents a sampling of the 2-ml sample) in an SW41 rotor (35,000 rpm for 18 h). Fractions (200 μl each) corresponding to visible bands at the 5/35% interface, were collected and washed raft fractions were made up to 2-ml volume again with 5 ml of isolation medium and 60% sucrose, and then adjusted to 45% sucrose. This suspension was then homogenized with 10 strokes in a loose-fitting Dounce, subjected to three 10-s bursts of homogenization in a sonicator (Tekmar sonicator). The homogenate was then adjusted to 45% sucrose and 0.25 M Na2CO3 (16) and subjected to equilibrium flotation in a sucrose gradient containing 0.25 M Na2CO3 throughout and layers of 3 ml of 5% sucrose, 4 ml of 35% sucrose, and the bottom load of 4 ml of 45% sucrose. The membrane raft band, which corresponds to the visible band at the 5/35% interface, was collected, diluted with ELB, and resedimented, and the pellet was resuspended in 100 μl of ELB.

In Figs. 3–5, plasma membrane rafts were derived using the Triton X-100 flotation procedure essentially as described by Lafont and Si-monas (17). Briefly, washed whole Hep3B cells (Figs. 3 and 5) or the washed P0-15 membrane fraction (Fig. 4) was made up to 0.05% Triton X-100 in solubilizing buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol) and incubated for 30 min at 4 °C. The extract was then adjusted to 60% sucrose in solubilizing buffer and subjected to equilibrium flotation in a sucrose gradient with layers of 1 ml of ELB, 1 ml of 13% sucrose, 2 ml of 43% sucrose, and 2.4 ml of the 60% sucrose plus sample in an SW50.1 rotor. The only visible band was now at the 43/60% sucrose interface; this was collected, diluted in ELB, resedi-

In Fig. 2B, plasma membrane fractions a and b in Fig. 2B (i) were derived from Hep3B cells by equilibrium flotation using a method modified from Greenawalt (14) and Aronson and Touster (15). Briefly, the Dounce-homogenized cell homogenate of Hep3B cells in ELB was subjected to two rounds of low speed centrifugation to remove nuclei, and then adjusted to 70 ml sucrose, 220 mM mannitol, and 2 mM Hepes, pH 7.4 ("isolation medium") (2.8 ml for extract derived from fifteen 100-mm cultures). This cytoplasmic extract was then subjected to cen-
refraction and isolation medium and 60% sucrose (design-

In Fig. 2B (ii) the washed flotation bands at the 13/43% sucrose interface in a and b in Fig. 2A (i and b) were pooled, treated with 0.1% saponin for 30 min on ice, adjusted to 60% sucrose in ELB and re-floated to equilibrium up a second sucrose gradient with layers of 1 ml of 13% sucrose, 2 ml of 43% sucrose, and −2.4 ml of the 60% sucrose plus sample in an SW50.1 rotor. The only visible band was now at the 43/60% sucrose interface; this was collected, diluted in ELB, resedi-

In Figs. 3–5, plasma membrane rafts were derived using the Triton X-100 flotation procedure essentially as described by Lafont and Simona (17). Briefly, washed whole Hep3B cells (Figs. 3 and 5) or the washed P0-15 membrane fraction (Fig. 4) was made up to 0.05% Triton X-100 in solubilizing buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol) and incubated for 30 min at 4 °C. The extract was then adjusted to 60% sucrose in solubilizing buffer and subjected to equilibrium flotation in a sucrose gradient with layers of 1 ml of ELB, 1 ml of 13% sucrose, 2 ml of 43%, and 2 ml of 60% (the sample) in an SW50.1 rotor (35,000 rpm for 18 h). Fractions (200 μl each) corresponding to visible bands were collected and washed raft fractions were made up to 2-ml volume again with 5 ml of isolation medium and 60% sucrose, and then adjusted to 45% sucrose and 0.25 M Na2CO3 (16) and subjected to equilibrium flotation in a sucrose gradient containing 0.25 M Na2CO3 throughout and layers of 3 ml of 5% sucrose, 4 ml of 35% sucrose, and the bottom load of 4 ml of 45% sucrose. The membrane raft band, which corresponds to the visible band at the 5/35% interface, was collected, diluted with ELB, and resedimented, and the pellet was resuspended in 100 μl of ELB.

In Figs. 3–5, plasma membrane rafts were derived using the Triton X-100 flotation procedure essentially as described by Lafont and Simona (17). Briefly, washed whole Hep3B cells (Figs. 3 and 5) or the washed P0-15 membrane fraction (Fig. 4) was made up to 0.05% Triton X-100 in solubilizing buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol) and incubated for 30 min at 4 °C. The extract was then adjusted to 60% sucrose in solubilizing buffer and subjected to equilibrium flotation in a sucrose gradient with layers of 1 ml of ELB, 1 ml of 13% sucrose, 2 ml of 43%, and 2 ml of 60% (the sample) in an SW50.1 rotor (35,000 rpm for 18 h). Fractions (200 μl each) corresponding to visible bands were collected and washed raft fractions were made up to 2-ml volume again with 0.05% Triton X-100 and solubilizing buffer and 60% sucrose, and subjected to equilibrium flotation in a shallow sucrose gradient with layers of 1 ml of 10% sucrose, 0.7 ml of 30%, 0.7 ml of 40%, 0.7 ml of 45%, and 2 ml of 60% sucrose in an SW50.1 rotor. Visible bands corresponding to fraction 1 located just above the 30/40% interface, fraction 2 (the main band) at the 30/40 interface, fraction 3 just below the 30/40
interface, and fraction 4, and the pellets were collected, diluted in ELB, resealed, and resuspended in 50 μL of ELB.

Enzymatic assays for 5′-nucleotidase (5′-ND, a marker for plasma membrane) and lactate dehydrogenase (LDH, a marker for cytosol) were carried out using kits obtained from Sigma Chemical Co. (St. Louis, MO). Additional Western blottable organelle markers used included very late antigen-2a (VLA-2a) for plasma membrane, caveolin-1, and flotillin-1 for caveolae and rafts, glucose-regulated protein 78 (GRP78/BiP) for endoplasmic reticulum, and nuclear pore-associated protein 62 (Nup62) for nuclei (Organelle Marker kit, Transduction Laboratories, Lexington, KY).

Western Blot Analyses of Proteins

Western blotting was carried out using 7.5% polyacrylamide-SDS gels under reducing denaturing conditions in accordance with procedures and protocols provided by Transduction Laboratories, Lexington, KY, and the ECL detection kit (Amersham Biosciences, Inc., UK) (9, 13). For quantitation purposes extensive previous calibration controls (9, 13) have shown that detection of STAT1 and STAT3 proteins and their Tyr-phosphorylated derivatives was linear with the amount of respective protein in this assay. Routinely, multiple exposures of each blot were obtained so as to ensure that each of the signals was within the linear range; and, to reduce quantitation errors, sample volumes in different fractions were adjusted to provide signals of equivalent strength each within the linear range of the assay. Western blot signals were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The reliability of STAT quantitation was additionally verified using the Molecular Dynamics Storm PhosphorImager system. Reagents for PhosphorImager Quantitation were purchased from Amersham Biosciences, Inc. and were used in accordance with procedures outlined in the reagent kit protocol. Briefly, the procedure was the same as for the peroxidase-based Western blot ECL method cited above except that an alkaline phosphatase-linked secondary antibody was used instead. The signals were amplified and detected by the Vistra ECF substrate and scanned and quantitated by phosphorimaging (9).

DNA Gel-shift Assays

STAT-specific DNA binding activity was assayed using the mutant m67 “SIE” oligonucleotide derived from the c-fos promoter (purchased from Santa Cruz Biotechnology, Santa Cruz, CA) as described earlier (9, 13). Briefly, this oligonucleotide yields the typical pattern of SIF-A, -B, and -C complexes in gel-shift assays corresponding to the STAT3 homodimer, STAT1/3 heterodimer, and STAT1 homodimer, respectively (9). The SIE oligonucleotide was 32P-end-labeled at its 5′-end using T4 polynucleotide kinase and used in electrophoretic shift assays as described earlier (9, 13). Typically, the binding buffer (15-μL reaction volume, 30-min incubation at room temperature) contained 40 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 20 mM Hepes, pH 7.9, 4% (v/v) Ficoll, and 1 μg Poly(dI-dC)/Poly(dI-dC) (Amersham Biosciences, Inc.). DNA-protein complexes were separated by electrophoresis through 5% PAGE (acrylamide/bisacrylamide ratio 30:0.8) in 0.2× TBE (0.02 mM Tris, pH 8.3, 0.02 M boric acid, 0.25 mM EDTA) at 300 V at 4 °C for ~2 h, and dried for autoradiography. As controls in the DNA-shift experiments shown in Figs. 1, 3, and 5, we have used the S100 cytosol from IL-6-treated Hep3B cells; in these illustrations the autoradiograms showing the concentration range from 0.1% to 1.0% and Triton X-100 at 0.05% did not affect the DNA binding assay for activated STATs (data not shown).

Antibody Reagents

Murine monoclonal antibodies to STAT1 and STAT3 were purchased from Transduction Laboratories (Lexington, KY), and rabbit polyclonal antibodies (pAbs) to STAT3 (H-190 and H-190X), STAT5b, the IL-6 receptor β chain gp130, the interferon (IFN)-γ receptor α chain IFN-γRα and c-myc9-1, as well as a murine monoclonal antibody to PY-STAT3 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-PY-STAT1 pAb was purchased from New England Biolabs, Beverly, MA. Antibodies to the organelle markers BiP, VLA-2a, Nup62, and PY-caveolin-1 were purchased from Transduction Laboratories. Rabbit antiserum to recombinant human glucose-regulated protein 58 (GRF58/ER60/ERP57) was a gift from Drs. Mohammed Boudri and Lance Pohl (National Institutes of Health, Bethesda, MD).

RESULTS

STAT3 Pre-associated with Cytosplasm Membrane Fractions—STAT proteins are customarily considered to be latent cytosolic transcription factors residing exclusively in the free cytosolic compartment (5–8, 18). Upon induction by cytokines, a portion of the cytosolic pool of STATs is thought to be activated by Tyr phosphorylation, and 15–25% of the cytosplasmic pool of the respective STAT species eventually translocates to the nucleus (9, 19). In earlier cell fractionation studies we observed that ~5–10% of cellular STAT3 appeared to be associated with a post-nuclear fraction sedimenting at 15,000 × g, which consisted of cytoplasmic membranes by electron microscopy (Fig. 2 in Ref. 9,2 the P0-15 fraction). Another ~5–10% of STAT3 was associated with a subsequent fraction sedimenting at 100,000 × g, which was free of cellular membranes as assayed by electron microscopy (Fig. 2 in Ref. 9, 2 the P100 fraction).

2 P. B. Sehgal, G. G. Guo, M. Shah, K. Patel, H. Shukla, W. Murray, and V. Fried, submitted for publication.
activity data show that approximately two-thirds of the plasma membrane marker (5'-ND) was recovered in the P0-15 fraction, whereas over 90% of the cytost marker (LDH) was recovered in the S100 cytost fraction. Although the P0-15 fraction was enriched in 5'-ND activity, this fraction contained very little cytostic LDH activity (Fig. 1A). The upper portion of Fig. 1A confirms our previous observation (9) that the P100 and P0-15 fractions contained substantial amounts of STAT3 and PY-STAT3. The present report is focused on evaluating the nature of the cellular membranes with which STAT3 and PY-STAT3 were associated in the P0-15 fraction. A separate report will discuss characterization of the membrane-free sedimentable complexes of STAT proteins present in the P100 cytostic fraction.2

In light of previous literature, which had apparently excluded the association of STATs with cellular membranes (18), we attempted to further evaluate whether the STAT proteins associated with the P0-15 membrane fraction in our experiments were derived from (i) contaminating cytost or (ii) nucleoprotein debris. Control and IL-6-treated Hep3B cells were fractionated with the objective of preserving the intactness of the nuclei and at the same time extensively washing the membrane fractions. Hep3B cells were homogenized in a loose-fitting Dounce, the nuclei removed by one round of low speed centrifugation of the homogenate, and a P0-15 pellet was obtained as described under “Materials and Methods.” The washed P0-15 pellet was gently resuspended in ELB, adjusted to 30% (w/v) sucrose, and then subjected to equilibrium centrifugation in a discontinuous sucrose gradient (Fig. 1, A and C). Seven visible bands and the pellet fraction were obtained. Fractions corresponding to fractions 3, 4, 5, and 6 were diluted in 40 volumes of ELB and again resedimented to yield the respective washed membrane fractions; the remaining fractions were analyzed on an as is basis. Fig. 1 (B and C) summarizes some of the data obtained. STAT3 was clearly associated with cytoplasmic membranes that sedimented in the vicinity of the 40%/60% sucrose interface (fractions 4–6). STAT3-containing fractions were enriched in the plasma membrane markers 5'-ND and VLA-2a, lacked any detectable cytostic LDH activity (data not shown; also see Fig. 1A) and could be distinguished from the sedimentation of the endoplasmic reticulum marker BiP. Little STAT3-specific DNA shift activity was observed; a trace amount of STAT1 (SIF-C) DNA shift activity was observed in membrane fractions from IL-6-treated cells (in fraction 4 in Fig. 1C); however, this activity was not in fractions corresponding to the bulk of the membrane-associated STAT3 in fractions 5 and 6 (Fig. 1C). STAT3, VLA-2a, and BiP in the pellet fraction in these gradients (fraction 8) represented the sedimentation of intact nuclei (together with the associated outer nuclear membrane, which is continuous with the endoplasmic reticulum) as evidenced by Western blotting using an anti-Nup62 antibody (data not shown). (In all subsequent fractionations the cell homogenate was subjected to two rounds of low-speed centrifugation to remove almost all the nuclear contamination.) The experiment in Fig. 1 (B and C) was replicated three times; thus, although in the example illustrated in Fig. 1 (B and C) there appeared to be STAT3 association with lighter membranes in fraction 4 in the IL-6-treated group, this result was also observed in the control group in additional replications. Overall, the data in Fig. 1 indicate that two-thirds of the cellular pool of the 5'-ND plasma membrane marker was recovered in the P0-15 pellet (Fig. 1A), and that in this membrane pellet fraction STAT3 was pre-associated with membranes with the same buoyant density as those containing the 5'-ND and VLA-2a plasma membrane markers (Fig. 1, B and C). These data in Fig. 1 are consistent with the further observations that, when fractions similar to those in fraction 6 of Fig. 1 (B and C) were analyzed by confocal two-color immunomicroscopy, STAT3 and the plasma membrane-associated protein TRAP1 (tumor necrosis factor receptor associated protein 1 [20]) colocalized in membrane fragments.3 Furthermore, quantitative two-color flow cytometry confirmed the colocalization of STAT3 with TRAP1 in membrane fragments.3

The association of STATs with plasma membrane and plasma membrane rafts was further evaluated using several different cell fractionation approaches. Fig. 2A shows that when the P2-15 membrane fraction from Hep3B cells was adjusted to 0.1% saponin and then subjected to equilibrium flotation, STAT1, STAT3, gp130, and 5'-ND activity was present in membranes with similar buoyant density (mainly flotation fraction 5). An alternative approach is illustrated in Fig. 2B. Plasma membrane fractions were first purified from control and orthovanadate-treated cells by equilibrium flotation into visible bands at the 13/43% sucrose interface derived from two separate sequential differential sedimentation steps (fractions a and b as described under “Materials and Methods”) (14, 15). The data in Fig. 2B (i) show pre-association of STAT1 and some STAT3 with 5'-ND-containing (i.e. plasma membrane) fractions and a vanadate-induced increase in association of STAT3, PY-STAT3, and STAT1. Additional Western blotting experiments showed that these fractions were devoid of the endoplas-
mic reticulum marker BiP (data not shown).

In the Western blot shown in Fig. 2B (i) only 10% of each fraction was assayed; the remainder of these fractions were pooled ("a" plus "b"), treated with saponin (0.1%), adjusted to 60% sucrose, and refloated to equilibrium. The visible plasma membrane and 5'-ND-containing band now appeared at the 43/60% interface. The data in Fig. 2B (ii), derived from a Western blot containing two-thirds of each of the re-floated fractions, show that the association of STAT3 and STAT1 with plasma membranes derived from control cells survived saponin-treatment. Although, saponin treatment of plasma membranes derived from vanadate-activated cells reduced the association of bulk STAT3 and STAT1 with rafts, substantial PY-STAT3 remained associated with membrane rafts (compare i and ii in Fig. 2B). None of the PY-STAT-containing raft fractions shown in Fig. 2B gave detectable STAT DNA binding activity (SIF-A and/or SIF-B DNA shift bands would have been expected from the amount of PY-STAT3 observed) (data not shown; also see below).

Fig. 2C shows that caveolin-1-containing plasma membrane rafts prepared from Hep3B cells using the non-detergent method of Song et al. (16) also yielded raft preparations that not only contained the integral raft protein caveolin-1 (and 5'-ND activity; data not shown) but also contained STAT1 and STAT3, and the previously reported STAT3-associated chaperone GRP58 (9). IL-6 treatment led to the appearance of PY-STAT3 in the caveolin-1-positive raft preparation obtained using this non-detergent method (Fig. 2C). However, this PY-STAT3 was also not accessible to DNA binding in a gel-shift assay (data not shown).

**STATs in Triton X-100-resistant Plasma Membrane Rafts—**

Simons and colleagues (17) recently reported a technique to investigate the role of detergent-resistant rafts in immunoglobulin E signaling, which involved the use of an isotonic solubilizing buffer containing 0.05% Triton X-100 to directly lyse cells followed by equilbrium flotation centrifugation. We have used this procedure to confirm the presence of STATs in detergent-resistant rafts and to obtain an estimate of the amount of cytoplasmic STATs and PY-STATs present in the raft fraction. Hep3B cells were treated with orthovanadate as a general activator of STATs, the cells were harvested, and the raft preparation was obtained. In Fig. 3, fraction 6 corresponds to the region in the sucrose gradient where the sample was loaded. All fractions other than fraction 6 were resedimented and resuspended in 100 μl of ELB. Western blot analyses represent 10% of each of fractions 1–5 and 7 (10 μl each) and 1% of fraction 6 (20 μl aliquot from the center of the 2-ml loading zone). Cytosol (S100 fraction) from IL-6-treated Hep3B cells was used as an internal control in DNA shift assays.

**Statistical Methods**

Statistical analyses were performed using the Student’s t-test. Differences were considered significant at a probability level of 0.05 (p < 0.05).

**Results**

**A. Blot**

Table 1 presents the results of the DNA-binding assays for STATs in Triton-resistant rafts prepared from whole cells. Control and orthovanadate-treated Hep3B cells (15 cultures per group) were harvested and treated with Triton X-100 (0.05%), adjusted to 60% sucrose (2 ml volume) followed by equilbrium flotation using the method of Lafont and Simons (17). Fraction 6 (2 ml) corresponds to the loading zone in the gradient where the sample was loaded. All fractions other than fraction 6 were resedimented and resuspended in 100 μl of ELB. Western blot analyses represent 10% of each of fractions 1–5 and 7 (10 μl each) and 1% of fraction 6 (20 μl aliquot from the center of the 2-ml loading zone). Cytosol (S100 fraction) from IL-6-treated Hep3B cells was used as an internal control in DNA shift assays.

**B. DNA Shift**

**C. 5'ND (%)**

Fig. 3. **STATs in Triton-resistant rafts prepared from whole cells.** Control and orthovanadate-treated Hep3B cells (15 cultures per group) were harvested and treated with Triton X-100 (0.05%), adjusted to 60% sucrose (2 ml volume) followed by equilibrium flotation using the method of Lafont and Simons (17). Fraction 6 (2 ml) corresponds to the loading zone in the gradient where the sample was loaded. All fractions other than fraction 6 were resedimented and resuspended in 100 μl of ELB. Western blot analyses represent 10% of each of fractions 1–5 and 7 (10 μl each) and 1% of fraction 6 (20 μl aliquot from the center of the 2-ml loading zone). Cytosol (S100 fraction) from IL-6-treated Hep3B cells was used as an internal control in DNA shift assays.

**Triton Flotation: P0-15 Membranes**

Table 2 presents the results of the DNA-binding assays for STATs in Triton-resistant rafts prepared from isolated membrane fractions. The P0-15 membrane fraction derived from control or orthovanadate-treated Hep3B cells (12 cultures per group) were treated with Triton X-100 (0.05%) and then plasma membrane rafts prepared using the method of Lafont and Simons (17) as in Fig. 3. Each fraction was resuspended in 100 μl of ELB except for fraction 6 (the 2-ml loading zone), and aliquots Western blotted (25 μl for fractions 2–5 and 7 and 50 μl for fractions 1 and 6) and assayed for 5'-ND activity (5 μl each).

**Fig. 4.** **STATs in Triton-resistant rafts prepared from isolated membrane fractions.** The P0-15 membrane fraction derived from control or orthovanadate-treated Hep3B cells (12 cultures per group) were treated with Triton X-100 (0.05%) and then plasma membrane rafts prepared using the method of Lafont and Simons (17) as in Fig. 3. Each fraction was resuspended in 100 μl of ELB except for fraction 6 (the 2-ml loading zone), and aliquots Western blotted (25 μl for fractions 2–5 and 7 and 50 μl for fractions 1 and 6) and assayed for 5'-ND activity (5 μl each).

Fig. 4 shows the preparation of STAT-containing Triton-resistant rafts derived from the P0-15 membrane fraction of Hep3B cells. In this experiment, because the membrane fraction had been previously extensively washed with ELB buffer prior to the Triton flotation step, there was little contamination by soluble cytosolic STAT proteins. This is evidenced by the absence of any detectable STAT proteins in fraction 6 (the loading zone in the sucrose gradient). The data in Fig. 4 show that almost all STAT1 and STAT3 detected was in Triton-resistant flotation rafts. These fractions also included the plasma membrane marker 5'-ND, the integral raft proteins caveolin-1 and flotillin-1, and the cytokine receptor chains gp130 and IFN-γRα. As would have been expected from previous studies (21), we were able to confirm that orthovanadate...
Western blot analyses were carried out using 10%/H9262 fraction 4 60% sucrose followed by equilibrium flotation using the method of were harvested and treated with Triton X-100 (0.05%), then adjusted to A cells.

Fig. 5. STATs in Triton-resistant rafts prepared from whole cells. A, control and IL-6-treated Hep3B cells (12 cultures sickle) were harvested and treated with Triton X-100 (0.05%), then adjusted to 60% sucrose followed by equilibrium flotation using the method of Lafont and Simons (16) as described under “Materials and Methods.” Western blot analyses were carried out using 10 μl of each fraction (which represented 20% of each of fractions 1–5 and 7 and 0.5% of the leading zone corresponding to fraction 8), and 5'-ND assays were carried out using 5-μl aliquots. Cytosol (S100 fraction) from IL-6-treated Hep3B cells was used as an internal control in DNA shift assays. B, the remainder of fractions 3–4 from each of the gradients shown in A were pooled, readjusted to 0.05% Triton X-100 and 60% sucrose, and subjected to re-flotation (layers of 10, 30, 40, 45, and 60% sucrose). Three visible bands in the 10–40% region (fractions 1–3) and the pellet (fraction 4) were collected, washed with ELB, resuspended in 50-μl volume, and analyzed (for Western blotting, 15 μl each; for 5'-ND, 5 μl each).

DNA shift
B. Triton Reflotation

FIG. 5. STATs in Triton-resistant rafts prepared from whole

treatment of Hep3B cells led to the appearance of PY-caveolin-1 in the STAT3- and STAT1-containing raft fractions.

STATs in Triton-resistant rafts were further investigated in IL-6-treated Hep3B cells. Fig. 5 shows an analysis of STATs in rafts obtained from control and IL-6-treated Hep3B cells that had been directly lysed in Triton and then subjected to equilibrium flotation (17). Fig. 5A shows that STAT3, STAT1, caveolin-1, and 5'-ND activity could be clearly observed in detergent-resistant flotation rafts (fractions 2–4). Upon IL-6 treatment there was little change in bulk STAT1 or STAT3 in IL-6-induced cells, the appearance of the STAT3 homodimer (SIF-C) complexes was markedly inhibited by MCD. Similiar in IFN-γ-treated cells, the appearance of the STAT3 homodimer (SIF-C) complexes were markedly inhibited by MCD. These data provide evidence for the functional dependence of cytokine-induced STAT signaling on the integrity of cholesterol-rich plasma membrane rafts.

STAT Signaling—The possible functional contribution of cholesterol-rich plasma membrane rafts to cytokine-induced STAT signaling was investigated by the use of the cyclodextrin derivative MCD. This compound when added to the culture medium binds to and sequesters cholesterol from the plasma membrane and has been used in numerous previous studies as a tool to disrupt cholesterol-rich plasma membrane rafts (2, 22). MCD inhibits raft-dependent signaling processes (2, 22). Hep3B cells were treated with MCD (12.5 mM) for 20 min and then with IL-6 or IFN-γ for another 15 min. The P100, S100, nuclear extract, and P0-15 fractions were obtained as illustrated in Fig. 5A, and STAT signaling was evaluated in DNA binding assays for activated STAT proteins. The DNA binding data in Fig. 5A show a marked inhibition of IL-6 and IFN-γ-induced STAT signaling into the P100, S100, and NE compartments. In IL-6-induced cells, the appearance of the STAT3 homodimer (SIF-A), STAT1/3 heterodimer (SIF-B), and the STAT1 homodimer (SIF-C) complexes were all markedly inhibited by MCD. Similarly in IFN-γ-treated cells the appearance of the STAT1 homodimer (SIF-C) complexes was markedly inhibited by MCD. These data provide evidence for the functional dependence of cytokine-induced STAT signaling on the integrity of cholesterol-rich plasma membrane rafts.

**DISCUSSION**

The critical first step by which cytokines exert their biological effects on cells is by activation of signaling pathways at the
level of the plasma membrane. Cytokine-activated STAT signaling eventually results in the translocation of Tyr-phosphorylated STAT family members to the nuclear compartment and the modulation of gene expression. Although the plasma membrane site is the first step in activation of STAT signaling, STAT activation at the level of the isolated plasma membrane fraction remains largely unexplored. Using cell fractionation and equilibrium flotation techniques we discovered that substantial amounts of inactive and activated STATs existed in detergent-resistant plasma membrane rafts derived from human hepatoma Hep3B cells. In these membranes, STATs co-rafted with the integral raft proteins caveolin-1 and flotillin-1, with cytokine receptor chains gp130 and IFN-γRα, and the chaperone GRP58. PY-STAT3 and PY-STAT1 were also associated with rafts but were deficient in DNA binding in this form. We suggest that this pool of PY-STATs represents a transient early stage in cytokine signaling prior to the departure of PY-STATs from the cytokine receptor. That the raft inhibitor MCD markedly reduced cytokine-induced STAT signaling to the cell interior adds further support to the hypothesis that STATs are activated by cytokines at the plasma membrane in specialized cholesterol-rich raft microdomains (the “raft-STAT signaling hypothesis”). Conceptually, the raft-STAT signaling hypothesis suggests that membrane rafts containing STATs may represent physical sites for integrating the combinatorial effects of different cytokines and different activation pathways.

The first “integral” raft protein to be identified was caveolin-1, a target of the Src-tyrosine kinase (2–4). Integral/structural raft proteins now known include caveolin-1, -2, -3, flotillin-1, -2, and stomatin (1, 2). Structural and functional heterogeneity of plasma membrane rafts is a newly emergent theme in this field (1, 2). Plasma membrane rafts include specialized microdomains, which may be distinct from morphological caveolae (1–4). Biochemical and morphological experiments have shown that a variety of signaling molecules are concentrated within these plasma membrane raft microdomains, such as Src family tyrosine kinases, Ha-Ras, endothelial nitric-oxide synthase, and heterotrimeric G proteins (1–4). Caveolin-1 and caveolae have been hypothesized to serve as compartmentalized signaling molecules and facilitate cross-talk among signaling cascades (the caveola signaling hypothesis) (3, 4). Insulin, urokinase, and bradykinin signaling have also been suggested to involve low density caveolin-1-containing flotation rafts (22–24). Proteins shown to interact with caveolin-1 include epidermal growth factor receptor, platelet-derived growth factor receptor, Ha-Ras, c-Src, and phosphatidylinositol 3-kinase (reviewed in Refs. 2, 3, 25, 26).

Overall, the present data show that STATs, various cytokine receptor chains, and the raft proteins caveolin-1 and flotillin-1 co-fractionate with membrane raft fractions of similar buoyant density. Nevertheless, the data in Fig. 2B (i) (differences in STAT1 and STAT3 distribution in a and b), Fig. 4, left side (STAT proteins appear in fraction 4, which contained smaller amounts of caveolin-1 and flotillin-1 compared with fraction 3), and Fig. 5B (5’-ND in fraction 1 but little STATs, gp130, or caveolin-1) suggest that the issue of heterogeneity of STAT-containing membrane rafts needs to be explored further. In preliminary experiments using Protein-A-magnetic bead-panning techniques we have observed that anti-caveolin-1 pAb cross-immunoprecipitated STAT3. Magnetic bead-panning techniques also showed the inclusion of STAT3, STAT1, gp130, and caveolin-1 in the same detergent-resistant rafts. Enumeration of proteins present in specific rafts containing specific STATs is now amenable to a magnetic bead-panning assay using specific anti-STAT antibodies and a proteomics approach.

The present data show the steady-state pre-association of substantial amounts of STATs with the plasma membrane raft fraction (10% of total cytoplasmic STATs), and the association of cytokine- or tyrosine-phosphorylated PY-STAT3 and PY-STAT1 with the raft fractions in perhaps an early transient form not readily accessible to DNA binding probes. In as much as the amount of STATs, which translocate from the cytoplasm to the nucleus in Hep3B cells in response to cytokines is in the range 15–25% (9, 26), it may well be that, in the interests of signaling efficiency, STATs that translocate to the nucleus preferentially derive from the pool of STATs pre-associated in membrane rafts as pre-assembled signaling complexes. The present data showing the sustained association of PY-STATs with rafts through several purification steps also suggest that Tyr phosphorylation may not in itself be sufficient for the departure of activated STATs from the plasma membrane.

The observation that a brief pretreatment of Hep3B cells with the cytochalasin MCD markedly inhibited IL-6- and IFN-γ-induced STAT signaling to the cell interior provides evidence for the functional role of cholesterol-rich plasma membrane rafts in cytokine-induced STAT signaling. Very recently it has been reported that treatment of cultured human fibroblasts with sphingomyelinase activated the DNA binding activity of STAT1 and STAT3 in nuclear extracts, suggesting that endogenous ceramide generated at the plasma membrane could activate STAT signaling (27). This sphingomyelinase-activated STAT signaling was also inhibited by MCD (27), an observation consistent with our findings of STATs in plasma membrane rafts. Signaling through specialized cholesterol-rich raft microdomains representing supramolecular assemblies of cytokine receptors, associated signaling molecules, and chaperones (9–13, 28–31) may well prove to be a general mechanism operating at the level of the plasma membrane through which cytokines and growth factors activate various STAT species in different cell types (the raft-STAT signaling hypothesis). Although the issues of raft heterogeneity with respect to STATs and distinctions between STAT signaling mechanisms involving lipid rafts versus involving specialized caveolae (2, 32) remain to be addressed, the present article provides experimental evidence for the association of substantial amounts of cytoplasmic STAT proteins with detergent-resistant membrane rafts and the functional contribution of STATs in rafts to cytokine-activated STAT signaling.

On an historical note, Tamm and colleagues (33, 34) reported observations in 1981, which are consistent with the raft signaling hypothesis for cytokines. These investigators probed the fluidity of the plasma membrane lipid bilayer of HeLa cells treated with IFN-β using electron spin resonance probes. IFN caused an increase in plasma membrane rigidity within 30 min (the earliest time point that was tested), which returned to control levels in 3–5 h. Tamm and colleagues (33, 34) inferred that “the early and transient change probably is related to signal generation and transmission.” Recent investigators have confirmed that attachment of extracellular ligands to raft microdomains increases the rigidity of phospholipids in the raft as assayed using fluorescence transfer techniques (reviewed in Refs. 1, 2 and references cited therein). We believe that the electron spin resonance studies of Tamm and colleagues (33, 34) anticipated our present understanding of cytokine-induced signaling through plasma membrane rafts.

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4 M. Shah, K. Patel, and P. B. Sehgal, unpublished data.
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