Evidence for a Role of ADAM17 (TACE) in the Regulation of Platelet Glycoprotein V*

Received for publication, January 3, 2005, and in revised form, February 2, 2005
Published, JBC Papers in Press, February 3, 2005, DOI 10.1074/jbc.M500041200

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Glycoprotein V (GPV) is a subunit of the GPIb-IX-V receptor for von Willebrand factor and thrombin and has been shown to modulate platelet responses to the two strongest physiological agonists, thrombin and collagen. Thrombin directly cleaves GPV from the platelet surface, yielding a 69-kDa fragment GPV f1 of unknown function. We show here that a ~82-kDa fragment of GPV is shed from the platelet surface upon cellular activation with phorbol 12-myristate 13-acetate or the collagen-related peptide. This shedding was inhibited by the broad range metalloproteinase inhibitor GM6001, the two potent ADAM17 inhibitors GW280264X and TAPI-2, and was absent in mice lacking functional ADAM17 (ADAM17 lacking Zn-binding domain; ADAM17Zn). Furthermore, we show that recombinant ADAM17 ectodomain efficiently releases GPV from the platelet surface. GPV is known to be associated with the intracellular regulatory protein calmodulin, which has previously been shown to be involved in ADAM17-mediated shedding of L-selectin from the surface of leukocytes. As in these reports, inhibition of calmodulin led to rapid GPV shedding from the platelet surface, a process that was again blocked by GM6001 or ADAM17 inhibitors and that was absent in ADAM17Zn mice. Inhibition of outside-in signaling through GPIb/IIa did not significantly affect GPV shedding, excluding an essential role of this pathway for the regulation of ADAM17 activity. These results demonstrate that GPV is cleaved upon agonist-induced platelet activation and show that ADAM17 is the major enzyme mediating this process.

Platelet adhesion and aggregation at sites of vascular injury is crucial to limit post-traumatic blood loss but may also harm tissue by occluding diseased vessels. The membrane glycoprotein (GP) Ib-IX-V complex plays a central role in these events in that it mediates the initial platelet tethering to the damaged vessel wall under conditions of elevated shear by interacting with collagen-bound von Willebrand factor. The receptor complex consists of four distinct polypeptides, GPIbα (Mr = 143,000), Ibβ (Mr = 22,000), V (Mr = 83,000), and IX (Mr = 20,000), in a 1:1:0.5:1 stoichiometry with ~25,000 copies per platelet. GPs Ib, V, and IX are structurally related and belong to the family of leucine-rich glycoproteins. The receptor complex is associated with the regulatory cytoplasmic protein calmodulin, which is involved in the free Ca2+ uptake upon platelet activation (3), but its exact role in GPIb-IX-V function is unclear.

The importance of the GPIb-IX-V complex is emphasized by the study of the Bernard-Soulier syndrome, an inherited bleeding disorder in which the complex is congenitally missing or dysfunctional because of mutations in the genes encoding GPIbα or GPIX (2). Likewise, targeted deletion of the GPIbα (4) or GPIbβ (5) genes in mice reproduces the Bernard-Soulier phenotype, as characterized by thrombocytopenia, giant platelets, and massively prolonged bleeding times. Although the essential role of GPIb-IX for normal platelet function is firmly established, the function of GPV in the receptor complex is only partly understood. GPV is merely loosely attached to GPIb-IX and, in contrast to GPs Ib and IX, is not essential for the expression of the receptor complex on the platelet surface. By contrast, GPV and IX are required for expression of GP V (2). GPV-deficient mice have normal platelet size and counts, and the cells display an unaltered von Willebrand factor binding activity (6, 7).

Nevertheless, recent studies have revealed that GPV plays a role in different pathways of platelet activation. On the one hand, GPV was found to serve as a collagen receptor and to facilitate GPV-dependent platelet-collagen interactions (8). On the other hand, GPV seems to act as a negative regulator of thrombin-induced platelet activation, in that cleavage of the protein unmask GPV as well as releasing 75- and 82-kDa fragments, respectively, but the significance of these observations is unresolved.

Proteolysis of membrane proteins is a well-characterized regulatory process in a number of systems, including adhesion molecules, cytokines, growth factors, and their receptors. In most cases, shedding of ectodomains is mediated by membrane-anchored metalloproteases (reviewed in Refs. 10–12). Calmodulin has been shown to play a role in shedding of L-selectin.
from leukocytes (13), a process that is mediated by tumor necrosis factor-α-converting enzyme (ADAM17), which belongs to the ADAM (a disintegrin and metalloproteinase) family of zinc-dependent proteinases. The importance of ADAM17-mediated ectodomain shedding is highlighted by the perinatal lethality of mice lacking the functional enzyme (14).

In the current study, we investigated the regulation of GPV in human and mouse platelets. We show that agonist-induced platelet activation or inhibition of calmodulin results in metalloproteinase-dependent shedding of an 82-kDa fragment of the receptor from the platelet surface. Furthermore, using mice lacking functional ADAM17 (ADAM17 lacking Zn-binding domain; ADAM17ΔZn/ΔZn), we identify ADAM17 as the responsible sheddase.

**EXPERIMENTAL PROCEDURES**

**Mice**—NMRI wild-type mice (6 to 10 weeks of age) were obtained from Charles River Laboratories (Sulzfeld, Germany) and kept in our animal facilities. ADAM17ΔZn mice (14) were kindly provided by Dr. Roy Black at Amgen (Seattle, WA) and crossed to obtain ADAM17ΔZn/ΔZn mice.

**Reagents**—EZ-Link sulfo-NHS-LC-biotin (Pierce), Tetramethylbenzidine (Europa Bioproducts Ltd, Cambridge, UK), Sepharose G (Amersham Biosciences), ADP, phosphol 12-myristate 13-acetate (PMA), high molecular weight heparin (Sigma), α-thrombin (Roche Molecular Biochemicals), the broad range metalloproteinase inhibitor GM6001, the calmodulin inhibitors W7 and W13 (Calbiochem), human recombinant tumor necrosis factor-α-converting enzyme (ADAM17; R&D Systems, Minneapolis, MN), hirudin (Roche Diagnostics), streptavidin-HRP (DakoCytomation Denmark A/S, Glostrup, Denmark), ECL solution (Amersham Biosciences), and sulfuric acid (Applichem, Darmstadt, Germany) were purchased. Collagen-related peptide (CRP) was kindly provided by Steve Watson, and the specific metalloproteinase inhibitors GM6001 and W13 were kindly provided by GlaxoSmithKline (Stevenage, UK).

**Antibodies**—JON/A, which preferentially binds to activated murine GPIb/IIb/IIIa was purchased from emfret Analytics (Wurzburg, Germany). Anti-human GPV and anti-human GPIb were purchased from Immunotools (Friesoythe, Germany). All other antibodies were generated, produced, and modified in our laboratories: p0p1 (anti-murine GPX), EDL1 (anti-GPIIIa) and 250/108 (anti-human GPIb), and p0p1 (anti-human GPIbβ) (15, 16).

**Platelet Preparation**—Mice were bled under ether anesthesia from the retro-orbital plexus. Blood was collected in a tube containing 10% (v/v) 1 μM sodium citrate or 7.5 units/ml heparin, and platelet-rich plasma (PRP) was obtained by centrifugation at 300 × g for 10 min at room temperature (RT). For washed platelets, PRP was centrifuged at 1000 g for 10 min at room temperature and the pellet was resuspended twice in modified Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM NaHPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 5 mM glucose, and 0.35% bovine serum albumin, pH 6.6) in the presence of prostacyclin (0.1 μg/ml) and apyrase (0.02 units/ml). Platelets were then resuspended in the same buffer (pH 7.0; 0.02 units/ml apyrase) and incubated at 37 °C for at least 30 min before use. Isolated platelets did not show any signs of activation as shown by flow cytometry (staining for P-selectin and surface-expressed fibrinogen). Because ADAM17ΔZn/ΔZn mice die perinatally (14), blood was collected from mutant and control mice immediately after birth.

**Preparation of Washed Human Platelets**—Washed-platelet-rich plasma was prepared from TBS-EDTA anticoagulated blood obtained from aspirin-free healthy volunteers and platelets were washed by sequential centrifugation as described previously (17). The cells were finally suspended in Tyrode’s buffer and adjusted to 3 × 10⁹ platelets/ml.

**Platelet Activation**—Washed platelets were resuspended at a concentration of 5 × 10⁹ platelets/ml in Tyrode’s buffer with 1 μM CaCl₂. The activators and inhibitors were added at the following concentrations: 2.5 μg/ml CRP, 5 μM ADP, 0.1 μg/ml PMA, 0.2 units/ml thrombin, 100 μg/ml GM6001, 200 μM W13, 200 μM GM6001, and incubated for 10 min (or 2 h for W13) at 37 °C. Platelets were then directly centrifuged at 400 × g for 5 min to obtain cell-free supernatants for ELISA and immunoprecipitation or analyzed by flow cytometry.

**Platelet Treatment with Recombinant ADAM17**—Platelets were washed twice in Tyrode’s buffer in the presence of prostacyclin (0.1 μg/ml) and apyrase (0.02 units/ml) and finally resuspended in the absence of prostacyclin and apyrase to a concentration of 5 × 10⁹ platelets/ml. Recombinant ADAM17 (dissolved at a concentration of 100 μg/ml in 25 mM Tris, pH 9.0, containing 2.5 μM ZnCl₂ and 0.005% Brij according to the manufacturer’s instructions) was added to the platelet suspension at the indicated concentrations and incubated for 1 h at 37 °C. Thereafter, the cells were analyzed by flow cytometry.

**Flow Cytometry**—Washed platelets (2 × 10⁹ in Tyrode’s buffer and 1 mM CaCl₂) were incubated with fluorophore-conjugated monoclonal antibodies (10 μg/ml) for 10 min at RT, the reaction was stopped with 500 μl of PBS, and the samples were immediately analyzed on a FACSCalibur (BD Biosciences).

**Aggregometry**—To determine platelet aggregation, light transmission was measured using p2P (200 μl with 0.5 × 10⁹ platelets/ml) and washed platelets. Transmission was recorded on a Fibrinimeter 4 channel aggregometer (APACT Laborgeräte und Analysensysteme, Hamburg, Germany) over 10 min and was expressed as arbitrary units with 100% transmission adjusted with plasma. Platelet aggregation was induced by addition of PMA (0.1 μg/ml), CRP (2.5 μg/ml), U46619 (0.1 μM), and ADP (5 μM). Thrombin-induced aggregation was performed with washed platelets (200 μl with 0.5 × 10⁹ platelets/ml in Tyrode’s buffer, 1 μmol/liter CaCl₂). For GPIb/IIb inhibition, JON/A (50 μg/ml) was added 3 min before addition of the activators.

**ELISA**—To detect cleaved GPV molecule with the ELISA system, platelets were treated with the indicated activators and the supernatants (100 μl of 0.5 × 10⁹ platelets/ml) were centrifuged at 15,000 × g for 10 min centrifugation at 15,000 × g. For GPIb/IIb blocking, JON/A (50 μg/ml) was added 3 min before addition of the activators. Supernatants were transferred onto αGPV-coated (DOM1; 20 μg/ml) ELISA plates, incubated for 1 h at 37 °C, and washed three times with washing buffer (1 × PBS and 0.1% Tween). Secondary antibody (DOM2-HRP) was added at a concentration of 2.5 μg/ml followed by 1 h incubation at 37 °C and detected with tetramethylbenzidine. The reaction was stopped with 0.5 M sulfuric acid and analyzed by WinRead.

**Immunoprecipitation**—Washed platelets were surface-labeled with EZ-Link sulfo-NHS-LC-biotin (25 μg/ml in PBS) and subsequently incubated with different reagents/agonists for the indicated times and then centrifuged (2000 × g; 10 min). Supernatants were then collected and incubated with 10 μg of DOM1 (anti-GPV), p0p1 (anti-GP Ib), or EDL1 (anti-GPIIIa) and 25 μL of protein G-Sepharose (Euro- biences) overnight at 4 °C. Samples were separated by 12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with streptavidin-HRP (1 μg/ml) for 1 h after blocking. After extensive washing, biotinylated proteins were visualized by ECL.

**RESULTS AND DISCUSSION**

To study GPV regulation on the platelet surface, we stimulated human platelets with PMA (100 ng/ml), ADP (5 μM), or CRP (2.5 μg/ml) and determined surface levels of GPV by flow cytometry. CRP activates the cells through the GPV1c/2R/ chain complex (18), the major platelet collagen receptor (19). As a positive control, platelets were incubated with thrombin (0.1 units/ml), which directly cleaves the receptor. GPV was down-regulated in response to PMA to a similar extent as in the thrombin control, whereas a weaker effect was observed in response to CRP. In contrast, ADP, which is only a weak platelet agonist, did not induce significant down-regulation of GPV (Fig. 1A).

GPIb-IX-V can be down-regulated by ectodomain shedding of GPIbα and/or GPV by or internalization of the entire receptor complex (15, 20). To investigate whether the decrease in GPV surface expression is accompanied by the down-regulation of the entire GPIb-IX-V complex from the cell surface, we determined the surface levels of GPIbβ, because this subunit is not cleaved upon platelet activation. GPIbβ surface levels were only slightly decreased or unchanged in PMA- or CRP-stimulated platelets, respectively, indicating that down-regulation of GPV was rather the effect of shedding than internalization (Fig. 1B).

PMA- or CRP-induced shedding of GPV occurred independently of thrombin activity in that it was unaltered in the presence of the thrombin inhibitor hirudin (data not shown). In contrast, the broad spectrum metalloproteinase inhibitor GM6001 markedly blocked GPV shedding in response to PMA...
and CRP but not thrombin (Fig. 1C). Very similar results were obtained when GPV regulation was studied in murine platelets. In these cells, PMA induced virtually complete down-regulation of the receptor, whereas the effect of CRP was weaker and ADP did not alter GPV surface levels (Fig. 2A). GPIX surface levels remained virtually unchanged under all conditions, confirming that GPV shedding is not accompanied by down-regulation of the entire GPIbIX-V complex from the cell surface (Fig. 2A). As in human platelets, GM6001 almost completely inhibited GPV shedding in response to PMA or CRP but not thrombin (Fig. 2B). Immunoprecipitation experiments demonstrated that the metalloproteinase-generated fragment of GPV has a molecular mass of ~82 kDa and thereby differs significantly from GPV f1 (~69 kDa) released by thrombin (Fig. 2C). As a control, immunoprecipitations were also performed with an anti-GPIIIa antibody, which did not yield a band under any condition (Fig. 2C). These results demonstrated that platelet activation by thrombin or PMA or CRP induces limited proteolysis of GPV via different mechanisms. PMA- or CRP-stimulated cleavage involves a platelet-derived metalloproteinase and occurs near the transmembrane domain.

The strong effect of PMA on GPV shedding prompted us to test a possible role of ADAM17 in this process, because this sheddase is known to be potently induced by protein kinase C activators. Therefore, we first tested platelets for the expression of ADAM 17 by Western blot analysis. As shown in Fig. 3A, ADAM 17 (~130 kDa) was specifically detectable in whole cell lysates of both human and mouse platelets. Next, we examined the effect of the two potent ADAM17 inhibitors, GW280264X (GlaxoSmithKline) (21, 22) and TAPI-2 (Immunex/Amgen) (23, 24) on GPV down-regulation. Both compounds inhibited PMA- and CRP-induced GPV shedding in human and mouse platelets (Fig. 3B), suggesting that ADAM17 mediates ectodomain shedding of GPV. To test this directly, platelets were incubated with different concentrations of recombinant human ADAM17 ectodomain (rhADAM17), and GPV levels were determined by flow cytometry. Indeed, rhADAM17 dose-dependently downregulated GPV (Fig. 3C), but not GPIX or GPIIb/IIIa (not shown) from the platelet surface, and this effect was abrogated in the presence of GM6001. To confirm the role of ADAM17 in this process, platelets from mice lacking functional ADAM17 (ADAM17<sup>−/−</sup> Zn<sup>−/−</sup>) were activated with PMA or CRP, and surface levels of GPV were determined by flow cytometry. As shown in Fig. 4, no GPV shedding was observed in these mice, demonstrating that ADAM17 is the major sheddase that cleaves GPV.

Besides GPV, a number of other receptors, including t-selectin on leukocytes (25) and GPVI on platelets (26), are known to be associated intracellularly with calmodulin and to undergo rapid ectodomain shedding upon treatment with calmodulin inhibitors, such as W13 (27, 28). To examine the implication of calmodulin in ADAM17-dependent GPV shedding, platelets were incubated with vehicle or the calmodulin inhibitor W13 (200 μM), and GPV surface levels were determined at different time points. As shown in Fig. 5A, GPV was rapidly downregulated in the presence, but not in the absence, of W13 in both mouse and human platelets. Similar results were obtained with a second calmodulin inhibitor, W7 (data not shown). As with CRP- or PMA-stimulated platelets, W13-induced GPV shedding was inhibited in the presence of GM6001, GW280264X, or TAPI-2 (Fig. 5A and data not shown) and was absent in platelets from ADAM17<sup>−/−</sup> Zn<sup>−/−</sup> mice (Fig. 5B). Moreover, immunoprecipitation experiments showed the accumulation of the ~82-kDa variant of soluble GPV in the supernatant of W13-treated platelets (Fig. 5C). Together, these results indicate that calmodulin is a negative regulator of ADAM17-
FIG. 2. Metalloproteinase- and thrombin-mediated GPV cleavage leads to the generation of different soluble GPV variants. Washed mouse platelets were incubated with the indicated agonists for 15 min at 37 °C, stained with FITC-labeled anti-GPV or anti-GPIX antibody (A) in the presence or absence of GM6001 (B), and analyzed immediately on a FACS Calibur. The data shown are the mean ± S.D. of six separate experiments and are presented as percentage of mean fluorescence determined at t = 0. C, surface-biotinylated mouse platelets were incubated with the indicated agonists for 15 min at 37 °C in the absence or presence of GM6001 (100 μM). Thereafter, GPV and GPIIIa were immunoprecipitated from the supernatants of the cells. Immunoprecipitates were separated by SDS-PAGE under reducing conditions, blotted onto a polyvinylidene difluoride membrane, and visualized using streptavidin-HRP and ECL. The data shown are representative of three identical experiments.

FIG. 3. Inhibition of ADAM17 blocks GPV shedding from the platelet surface. A, whole platelet proteins were separated by SDS-PAGE under reducing conditions and immunoblotted with anti-ADAM17 antibodies. B, human (left) and mouse (right) platelets were activated in presence or absence of TAPI-2 (100 μM) or GW280264X (10 μM), stained with FITC-labeled anti-GPV antibody and analyzed immediately on a FACS Calibur apparatus. Data shown are the mean ± S.D. of six separate experiments and are presented as percentage of mean fluorescence determined at t = 0. C, washed mouse platelets were incubated with the indicated concentrations of recombinant human ADAM17 for 1 h at 37 °C, stained with FITC-labeled anti-GPV antibody and analyzed immediately on a FACS Calibur apparatus. Where indicated, the experiment was performed in the presence of GM6001 (100 μM). Data shown are the mean ± S.D. of three separate experiments.
mediated GPV cleavage. As described previously for L-selectin (13) dissociation of calmodulin from the cytoplasmic tail of GPV may prime the receptor by as-yet-undefined mechanisms for ADAM17-mediated proteolysis.

Besides GPV, a number of other platelet membrane glycoproteins can undergo ectodomain shedding on the surface of activated platelets, including P-selectin, CD40-L, and GPVI; it seems that different regulatory mechanisms and proteases may be involved in these processes. Shedding of CD40-L has been reported to be largely dependent on activated GPIIIb/IIIa, suggesting that outside-in signaling through the integrin regulates the proteolytic process (29, 30). In contrast, GPVI shedding occurs completely independent of GPIIb/IIIa signaling (26). To test the involvement of GPIIb/IIIa outside-in signaling in ADAM17-mediated GPV shedding, we stimulated mouse platelets with PMA, CRP, or thrombin under stirring conditions in the presence or absence of a blocking antibody against GPIIb/IIIa (JON/A, 50 μg/ml; Ref. 31). Cleaved GPV was quantitated in the supernatant with a newly established ELISA system. Although JON/A blocked platelet aggregation (Fig. 6A), it had no significant effect on GPV shedding in response to PMA, thrombin (Fig. 6B), or CRP (data not shown), demon-

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**Fig. 4.** **GPV shedding is abolished in ADAM17ΔZn/ΔZn mice.** Platelets from wild-type or ADAM17ΔZn/ΔZn mice were incubated in the absence (shaded area) or presence (black line) of the indicated agonists for 15 min at 37 °C, stained with FITC-conjugated anti-GPV antibody, and analyzed immediately on a FACScalibur. The results shown are representative of four independent experiments.
strating that this process occurs independently of outside-in signaling through GPIIb/IIIa.

Our results show that ADAM17 is expressed in platelets and demonstrate that it mediates ectodomain shedding of GPV. Similar to other ADAM17 substrates, GPV cleavage occurs near the transmembrane region, leading to the release of an intact GPV ectodomain. The significance of soluble GPV is still unknown, but its generation during platelet activation has been used to develop a specific ELISA for the receptor that may become useful for monitoring platelet activation under conditions of clinical thrombosis (32). This approach was initially based on the hypothesis that thrombin is the major protease that cleaves GPV from the platelet surface, therefore making soluble GPV a specific indicator of intravascular thrombin activity. However, our results show that platelet activation in the absence of thrombin results in ADAM17-mediated ectodomain shedding of GPV. The generated fragment is bigger than GPV 

Fig. 6. GPV shedding occurs independently of GPIIb/IIIa outside-in signaling. Washed mouse platelets (thrombin) or heparinized prp (PMA) were incubated with the indicated activators in absence or presence (+) of JON/A (50 μg/ml) and (A) light transmission was recorded on a Fibrintimer 4 channel aggregometer. B, GPV levels in the supernatants were determined by ELISA as described under “Experimental Procedures.” Results shown are the mean ± S.D. of six individual experiments.

Fig. 5. Inhibition of calmodulin induces metalloproteinase-dependent shedding of GPV in mouse and human platelets. A, washed human and mouse platelets were incubated in the presence or absence of GM6001, with W13 (200 μM) for 2 h at 37 °C, stained with FITC-labeled anti-GPV antibody, and analyzed immediately on a FACScalibur apparatus. The data shown are the mean ± S.D. of six separate experiments. B, washed platelets from wild-type or ADAM17−/− mice were incubated with W13 (200 μM) for 2 h at 37 °C, stained with FITC-labeled anti-GPV antibody, and analyzed immediately on a FACScalibur apparatus. The data shown are the mean ± S.D. of four separate experiments. C, surface-biotinylated mouse platelets were incubated with W13 (200 μM) for 2 h at 37 °C in presence or absence of GM6001 (100 μM). Thereafter, GPV and GPIIb/IIIa were immunoprecipitated from the supernatants of the cells. Immunoprecipitates were separated by SDS-PAGE under reducing conditions, blotted onto a polyvinylidene difluoride membrane, and visualized using streptavidin-HRP and ECL. The data shown are representative of three identical experiments.

ADAM 17 is a well known sheddase that cleaves a variety of receptors, including α-selectin, VCAM-1 (14, 33, 34), and, as shown very recently, platelet GPIIb (35). As in the regulation of these molecules, the release of GPV from the platelet surface can be stimulated by the phorbol ester PMA through activation
of ADAM17, a pathway that seems to be conserved across multiple cell types. This is also supported by the observation that calmodulin plays a central role in the regulation of the shedding of GPV and L-selectin. Calmodulin is known to bind to the endodomain of both receptors, and prevention of such interaction by calmodulin inhibitors stimulates ectodomain shedding (Fig 5) (13, 36). Very recent evidence suggests that similar mechanisms may be involved in the cellular regulation of the major platelet collagen receptor GPVI (26). Besides its interaction with receptors, calmodulin also acts as a cellular intermediate of multiple Ca\(^{2+}\) actions and acts to interfere with the protein kinase C pathway (37, 38). Although protein kinase C itself is a potent activator of ADAM17, protein kinase C pathway (37, 38).

In conclusion, we show that the endogenous metalloproteinase ADAM17 is the major sheddase for GPV on the platelet surface. The shedding process can be regulated via calmodulin inhibition and is independent of outside-in signaling through the GPIb/IIa complex. The physiological relevance of this shedding process needs to be further elucidated regarding both the impact of GPV shedding on the platelet itself and the biological properties of the GPV fragment released from the membrane.

Acknowledgment—We thank S. Hartmann for excellent technical assistance.

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