

# Exogenous Glycosyl Phosphatidylinositol-anchored CD59 Associates with Kinases in Membrane Clusters on U937 Cells and Becomes Ca<sup>2+</sup>-signaling Competent

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**Abstract.** CD59, an 18–20-kD complement inhibitor anchored to the membrane via glycosyl phosphatidylinositol (GPI), can induce activation of T cells and neutrophils upon cross-linking with antibody. GPI-anchored molecules cocluster in high mol wt detergent-resistant complexes containing tyrosine kinases that are implicated in the signaling pathway. Exogenous, incorporated GPI-anchored molecules are initially unable to induce activation, presumably because they are not associated with kinases. Here we demonstrate that erythrocyte-derived CD59 incorporated in a CD59-negative cell line acquires signaling capacity in a time-dependent manner. Confocal microscopy revealed an initial dif-

fuse distribution of CD59 that became clustered within 2 h to give a pattern similar to endogenous GPI-anchored molecules. Gel filtration of detergent-solubilized cells immediately after incorporation revealed that CD59 was mainly monomeric, but after 3 h incubation all was in high mol wt complexes and had become associated with protein kinases. Newly incorporated CD59 did not deliver a Ca<sup>2+</sup> signal upon cross-linking, but at a time when it had become clustered and associated with kinase activity, cross-linking induced a large calcium transient, indicating that CD59 had incorporated in a specialized microenvironment that allowed it to function fully as a signal-transducing molecule.

**M**ANY proteins are attached to the cell membrane via glycosyl phosphatidylinositol (GPI)<sup>1</sup> rather than by a transmembrane anchor and have no direct contact with the inside of the cell. Despite this, it has been shown on several cell types that perturbation of GPI-anchored proteins by binding a mAb followed by cross-linking with a second antibody rapidly induces cell activation as assessed by induction of Ca<sup>2+</sup> influx and release of Ca<sup>2+</sup> from stores, generation of reactive oxygen metabolites, inositol phosphate turnover, cytokine release, and mitosis (Robinson, 1991; Brown, 1993; Morgan et al., 1993). GPI-anchored molecules exist on the surface of nucleated cells in detergent-resistant clusters associated with tyrosine kinases of the src family, cholesterol, glycosphingolipids, and, in some cell types, with caveolin (Stefanova and Horejsi, 1991; Stefanova et al., 1991; Anderson, 1993;

Lisanti et al., 1994). Cell activation after cross-linking of GPI-anchored molecules probably is facilitated through interaction with the tyrosine kinases. These kinases are attached to the inner lamella of the membrane by acyl chains. Perturbation of the membrane by mAb binding followed by cross-linking with a second antibody activates the tyrosine kinases. It has been suggested that interaction of the GPI-anchored molecule with kinase requires a transmembrane-transducing element, the identity of which remains controversial (Lisanti et al., 1994).

CD59, an 18–20 kD GPI-anchored glycoprotein is abundantly expressed on a wide variety of cells where it acts to inhibit complement-mediated lysis by interfering with assembly of the membrane attack complex (MAC) (for review see Davies and Lachmann, 1993). Antibody cross-linking of CD59 and of other GPI-anchored molecules on neutrophils and lymphocytes induces a cascade of activation events (Robinson, 1991; Morgan et al., 1993). Nonlethal complement attack induces a similar series of events, including increases in intracellular-free calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) and reactive oxygen production (Morgan, 1989; Morgan et al., 1993). The similarities between these activation cascades, and the known close association of CD59 with the MAC has led us to propose that the MAC is the natural ligand for CD59 and some aspects

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1. *Abbreviations used in this paper:* CLSM, confocal laser scanning microscopy; GAM, goat anti-mouse; GPI, glycosyl phosphatidylinositol; MAC, membrane attack complex.

of MAC-induced cell activation are mediated through cross-linking of CD59.

To further investigate the role of CD59 in cell activation by the MAC, we have set out to examine the activities of CD59 incorporated in a CD59-negative subline of U937 (van den Berg et al., 1994) in complement-induced recovery and activation events. A problem with this approach is that we and others have previously found that incorporated GPI-anchored molecules, unlike those endogenous to the cell, do not mediate activation events upon cross-linking (Zhang et al., 1992; Morgan et al., 1993), perhaps because incorporated CD59 is not capable of associating with tyrosine kinases. Here we further investigate this observation by examining whether incorporated GPI-anchored molecules can, in time, associate with protein kinase activity and acquire  $\text{Ca}^{2+}$ -signaling capacity. To test this CD59 was incorporated into the CD59-negative U937 cell line and its fate followed over several hours using different techniques to assess its clustering, protein kinase association, and  $\text{Ca}^{2+}$ -signaling capacity. Here we report that exogenously added CD59, while initially diffusely distributed on the membrane and incapable of  $\text{Ca}^{2+}$ -signaling, becomes clustered in a time-dependent manner, associates in multimolecular complexes with protein kinases, and concomitantly acquires  $\text{Ca}^{2+}$ -signaling activity.

## Materials and Methods

### Cells

The CD59-negative promonocyte cell line U937 used in this study was obtained from the European Collection of Animal Cell Cultures (87010802; ECACC, Porton Down, Salisbury, UK), and has been previously described in detail (van den Berg et al., 1994). Cells were grown at 37°C and 5%  $\text{CO}_2$  in RPMI 1640 medium supplemented with 10% FCS, 4 mM glutamine, 2 mM sodium pyruvate, 100 IU/ml penicillin, 100 IU/ml streptomycin, 2.5  $\mu\text{g}/\text{ml}$  amphotericin, and kept at a density of  $10^5$ – $10^6$  cells/ml. Cells were washed three times in PBS before use.

### Buffers and Reagents

FACScan buffer: 1% BSA, 0.2% Na-azide in PBS; kinase buffer: 25 mM Hepes, 5 mM  $\text{MnCl}_2$ , 0.1% Brij58, pH 7.5; Krebs-Hepes buffer: 120 mM NaCl, 4.8 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 1.3 mM  $\text{CaCl}_2$ , 1 mg/ml BSA, 25 mM Hepes, pH 7.4; lysis buffer: 20 mM Tris, 2 mM EDTA, 140 mM NaCl, 5 mM iodoacetamide, 1% Brij58 (Pierce and Warriner LTD, Chester, UK), pH 8.2; PBS: 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , 137 mM NaCl, 2.7 mM KCl, pH 7.4 (Oxoid Ltd., Basingstoke, UK). CD59 was purified from human erythrocytes using an immunoaffinity column (MEM-43; Stefanova et al., 1989). CD59 was labeled with FITC using a published method (Johnstone and Thorpe, 1987), which resulted in a molar ratio FITC/CD59 of 2.6. CD59 was labeled with  $^{125}\text{I}$  (Amersham International, Amersham, U.K.) using immobilized chloramine-T (iodobeads; Pierce Chemical Co., Rockford, IL) resulting in a specific activity of  $2.4 \times 10^5$  cpm/ $\mu\text{g}$  protein. Neither FITC nor  $^{125}\text{I}$  labeling affected the complement inhibitory activity of CD59 (data not included). Filipin, obtained from Sigma Chemical Co. (Poole, Dorset, UK), was dissolved in DMSO at 5 mg/ml.

### Antibodies

Monoclonal antibodies were "in house" (Prague: MEM-43: anti-CD59; MEM-118: anti-CD55), or obtained from commercial sources: International Blood Group Reference Laboratory (IBGRL, Elstree, UK; BRIC229: anti-CD59; BRIC216: anti-CD55), Dako (High Wycombe, Bucks, UK; Goat anti-mouse (GAM)/IgG-FITC), Sigma Chemical Co. (GAM)/IgG-TRITC). GAM/IgG-HRP was obtained from Bio-Rad Laboratories (Hemel Hempstead, UK). GAM/IgG was obtained from Sigma Chemical Co.

### FACScan Analysis

Cells were harvested, washed three times with PBS, and resuspended at  $10^6$  cells/ml in FACS<sup>®</sup> buffer. All steps were carried out on ice. Cells were incubated with anti-CD59 (BRIC229: 2  $\mu\text{g}/\text{ml}$ ) for 30 min, washed three times with FACS buffer and incubated for 30 min with GAM/IgG-FITC. Cells were washed three times in FACS buffer and fixed with 1% paraformaldehyde in PBS. Fluorescence was measured using a FACScan (Becton-Dickinson and Co., San Jose, CA).

### Confocal Laser Scanning Microscopy (CLSM)

Cells were prepared for CLSM as follows: cells ( $10^7$ /ml) were incubated with 15  $\mu\text{g}/\text{ml}$  of fluorescent CD59 for 10 min at 37°C, washed in PBS, and fixed in 3% paraformaldehyde/0.5% glutaraldehyde for 1 h at room temperature (RT) or first incubated in medium for 2 h at 37°C and then fixed. In some experiments, cells ( $10^7$ /ml) were first incubated with CD59 (15  $\mu\text{g}/\text{ml}$ ) for 10 min at 37°C, incubated in medium for 2 h at 37°C, then filipin (50  $\mu\text{g}/\text{ml}$ ) was added and cells were incubated for another hour at 37°C. Cells were then stained for CD59 following the procedure described above using GAM/IgG-TRITC as the secondary antibody. Cells were stained for endogenously expressed DAF by the same protocol using mAb BRIC216 (2  $\mu\text{g}/\text{ml}$ ) and GAM/IgG-FITC and fixed in paraformaldehyde/glutaraldehyde. Cells were washed once in water and dried onto a microscope slide at 37°C. The slides were mounted in Citifluor (Citifluor Ltd., London, UK) and sealed. CLSM was carried out on a confocal microscope (TCS; Leica Inc., Deerfield, IL) using the  $\times 100$  oil immersion lens. FITC was excited at 488 nm and emission was measured above 515 nm. TRITC was excited at 568 nm and emission was measured above 590 nm. 12 sections were collected per field at regular intervals of 0.5–0.8  $\mu\text{m}$ . Sections were then either assembled as extended focus views or individual sections were viewed as a gallery.

### Incorporation of CD59

CD59, either unlabeled or labeled with FITC or  $^{125}\text{I}$ , was incorporated into the U937 cell line as follows: cells were harvested, washed three times in PBS, and CD59 (at the concentrations indicated) was added to cells at  $10^7$ /ml in PBS. After 10 min at 37°C, cells were washed with PBS and resuspended in the appropriate buffer or in complete medium (RPMI + additives). Cells incubated with  $^{125}\text{I}$ -labeled CD59 were resuspended at  $0.5 \times 10^6$  c/ml in RPMI and additives and incubated at 37°C and 5%  $\text{CO}_2$ . At different time points after incorporation 1-ml samples were taken, cells were spun (3 min at 725 g), and washed once with PBS.  $^{125}\text{I}$  was measured in the cell pellet and in the supernatant. In one set of experiments CD59 was incorporated into the U937 cells in the presence of 50  $\mu\text{g}/\text{ml}$  filipin. After 1 h incubation cells were analyzed as indicated.

### Kinase Assay, SDS-PAGE, and Western Blotting of Cell Lysates

**Preparations of Cell Lysates.** Cell lysates were prepared as described by Cinek and Horejsi (1992). Briefly, cells were resuspended in ice-cold lysis buffer at  $5 \times 10^7$ /ml. After 30 min incubation on ice, insoluble material was removed by centrifugation (10,000 g, 3 min, 4°C). The supernatant was used in further experiments.

**Gel Filtration of Cell Lysates.** Cell lysates were fractionated by gel filtration. Lysate (300  $\mu\text{l}$ ) was applied to a Sepharose 4B (Pharmacia, St. Albans, UK) column (4 cm  $\times$  0.7 cm), equilibrated and run in the lysis buffer at 4°C, and 300- $\mu\text{l}$  fractions were collected.

**Kinase Assay.** Kinase assay was carried out as described (Cinek and Horejsi, 1992). Briefly the wells of flexible polyvinylchloride 96-well microtiter plates (ICN-Flow, High Wycombe, Bucks, UK; Flow cat. No. 77-173-05) were coated with GAM/IgG (0.1 mg/ml, 1 h at 37°C) followed by incubation with specific monoclonal antibody (1–10  $\mu\text{g}/\text{ml}$ ; 4 h, 4°C). Antibodies used for immunoprecipitation were: MEM-43 or BRIC229 for CD59, MEM-118 or BRIC216 for DAF/CD55. Plates were washed and blocked with gelatin (2 mg/ml PBS, 1 h 37°C). The plates were incubated overnight at 4°C with cell lysate or fractions from the gel filtration column, the plates were washed thoroughly with cold PBS and  $^{32}\text{P}$ - $\gamma$ -ATP (Amersham International, Amersham, UK; sp. act. 5,000 Ci/mmol; 0.1  $\mu\text{Ci}/\text{well}$  in kinase buffer) was added. After a further incubation (25 min at room temperature) and washing, the immunoprecipitates were harvested in SDS solubilizing buffer, resolved on 10% SDS-PAGE gels (Laemmli, 1970) under reducing conditions, and the  $^{32}\text{P}$ - $\gamma$ -ATP-labeled proteins were detected by autoradiography.

**Western Blots.** Cell lysates and fractions off the gel filtration column were mixed 1:1 with electrophoresis sample buffer and run on a 15% gel under nonreducing conditions. After blotting onto nitrocellulose and blocking with 5% defatted milk in PBS, blots were incubated with mAb anti-CD59 (BRIC229 at 1  $\mu\text{g/ml}$  in 5% milk/PBS), washed with PBS/0.1% Tween 20, incubated with GAM/IgG-HRPO (1/2,000 in 5% milk/PBS), washed with PBS/0.1% Tween 20 and PBS. Blots were developed using the enhanced chemiluminescence technique (Amersham International) and visualized using x-ray film (Konica, Tokyo, Japan).

### Measurement of $[\text{Ca}^{2+}]_i$ during Cross-linking

U937 cells were incubated with CD59 (15  $\mu\text{g/ml}$ ) for 15 min at 37°C. Cells were washed and incubated in medium for 2 h washed and loaded with Fura-2 acetoxymethyl ester for 1 h at room temperature (Molecular Probes, Portland, OR) as described (Davies et al., 1991). Fura-2-loaded U937 cells ( $10^7/\text{ml}$ ) were incubated with the appropriate first antibody (20  $\mu\text{g/ml}$ , for 15 min at 4°C), washed and resuspended in Krebs-Hepes buffer. Cells were diluted 1/10 in Krebs-Hepes buffer in a stirred quartz cell in the chamber of the dual wavelength fluorometer (Spex Fluorolog; Glen Spectra, Stanmore, UK) at 37°C. After temperature equilibration, 0.5 mg of sheep anti-mouse IgG F(ab)<sub>2</sub> was added. Simultaneous excitation of entrapped Fura-2 at 340 and 380 nm was achieved using two lamps and monochromators together with a mirrored chopper system and fluorescence emission was monitored at 505 nm. The average  $[\text{Ca}^{2+}]_i$  in the cell population was calculated from the ratio of fluorescence emission at the two excitatory wavelengths, as previously described (Al-Mohanna and Hallett, 1988; Davies et al., 1991).

## Results

### Incorporation of CD59 in U937

Purified erythrocyte-derived human CD59 was incubated at various concentrations with the CD59-negative promonocytic cell line U937. At intervals after addition of CD59 cells were washed and prepared for FACscan analysis. CD59 incorporated very efficiently in the U937 cells in a dose- and time-dependent manner. Incorporation was stable in that multiple washes at 4°C failed to reduce the amount of CD59 bound. Incorporation was additionally confirmed by demonstrating CD59-mediated protection against complement lysis (data not included). Levels equivalent to or higher than the levels on CD59-positive cell lines (U937 subline; van den Berg et al., 1994) were easily obtained (Fig. 1 A). Incorporation was maximal after 15 min incubation. Retention of the incorporated CD59 on the surface of the U937 cells was measured in two ways using unlabeled CD59 or <sup>125</sup>I-labeled CD59. Af-

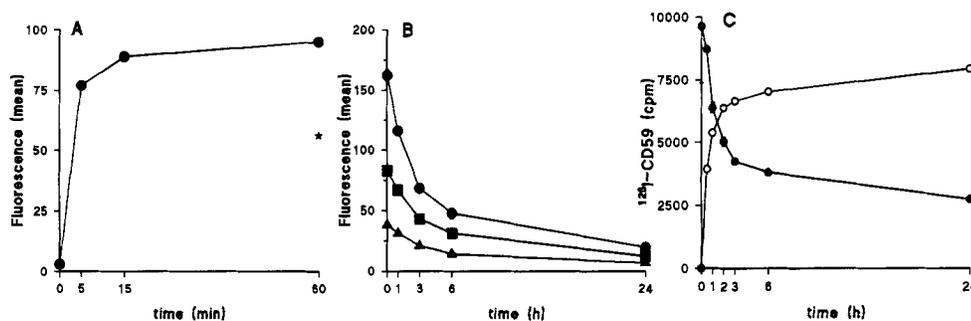
ter incubation cells were washed, resuspended in complete medium, and incubated at 37°C and 5% CO<sub>2</sub>. At various times after incorporation unlabeled CD59 was detected using BRIC229 on the FACscan (Fig. 1 B) and <sup>125</sup>I-labeled CD59 was detected on cells and supernatant after separation by centrifugation (Fig. 1 C). In both cases an initial rapid loss of CD59 from the cell surface (50% in 2 h) was followed by a second phase of slow removal (10–20% left after 24 h). Radioactivity appearing in the supernatant closely mirrored loss from the cells suggesting direct shedding. The close correlation between the two methods, one detecting only cell-surface CD59 and the other total cell-associated CD59, makes it unlikely that CD59 was internalized by the cells.

### Confocal Laser Scanning Microscopy

To exclude the possibility that antibody staining of the incorporated CD59 induced clustering, U937 cells were incubated with directly fluorescently labeled CD59 and prepared for CLSM. Cells were fixed immediately after incorporation of CD59-FITC, or, using an identical fixation protocol, after 2 h incubation in medium. Immediately after incorporation, CD59 was distributed evenly over the cell surface (Fig. 2, A–C). This is best seen in the gallery display of individual sections of the cell (Fig. 2, B and C). The slightly ruffled appearance is typical of membrane staining for this cell type. There was no fluorescence present inside the cells. After 2 h incubation in medium the distribution of the CD59 had dramatically altered and now was markedly clustered on the cell surface (Fig. 2, D–F), again, there was little or no fluorescence detectable inside the cells. This pattern closely resembled the distribution of endogenously expressed DAF/CD55 (stained with antibody) on the same cells (Fig. 2, G–I) and endogenous CD59 expressed on several other nucleated cells (Morgan et al., 1993; and our unpublished observations).

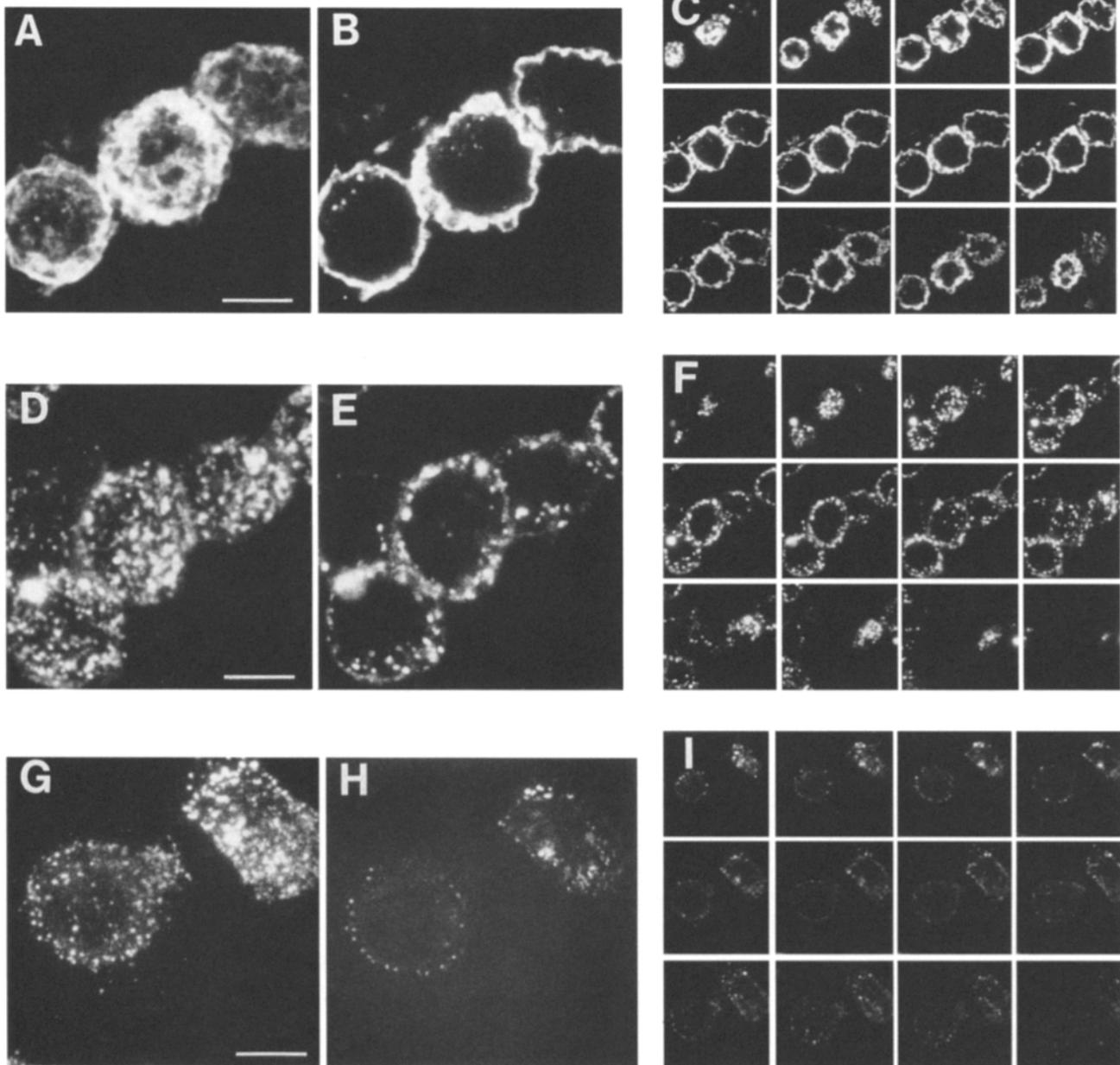
### Association with Other Molecules in Detergent-resistant Complexes

The clustering observed above might represent aggregation of CD59 alone or its association with other molecules in GPI-rich clusters. To establish if CD59 incorporated in cells became associated with other molecules in high mol



**Figure 1.** FACscan analysis of CD59 incorporated into U937. (A) U937 cells were incubated with 5  $\mu\text{g/ml}$  CD59 for various periods of time, washed, and stained for CD59 (●). ★, level of expression of CD59 in the CD59 positive subline (van den Berg et al., 1994). (B) U937 cells were incubated with CD59 (●: 15, ■: 5, ▲: 1.5  $\mu\text{g/ml}$ ) for 10 min. Cells

were washed and incubated in medium for various periods of time and stained for CD59 and analyzed on a FACscan. (C) U937 cells were incubated with <sup>125</sup>I-labeled CD59 (5  $\mu\text{g/ml}$ ) for 10 min. Cells were washed, incubated in medium for various periods of time, and <sup>125</sup>I was measured in the cell pellet (●) and supernatant (○). Results are expressed as means  $\pm$  SEM of experiments carried out in duplicates.



**Figure 2.** CLSM of U937 incubated with FITC-labeled CD59 and staining for endogenously expressed DAF. (A–F) U937 cells were incubated with FITC-labeled CD59 (15  $\mu\text{g/ml}$ ) for 10 min at 37°C. Cells were washed and fixed in 3% paraformaldehyde/0.5% glutaraldehyde at RT or incubated in medium for 2 h and then fixed. (A) extended focus immediately after incorporation of FITC-CD59. (B) frame 6 of gallery (C). (C) gallery of A. (D) extended focus 2 h after incubation with CD59. (E) frame 6 of gallery (F). (F) gallery of D. (G–I) U937 stained for endogenous DAF using mAb Bric216 and GAM/IgG-FITC; (G) extended focus. (H) frame 6 of gallery (I). (I) gallery of G. Bar, 10  $\mu\text{m}$ .

wt detergent-resistant complexes, cells were lysed in 1% Brij58 at various time points after incubation with CD59 and the lysates were subjected to gel filtration. Fractions were analyzed by Western blotting and kinase assays. Western blotting showed that CD59 immediately after incorporation in the U937 cells was mainly in the low mol wt fraction (Fig. 3 A, fraction 7–11). After incubation in medium at 37°C incorporated CD59 gradually became associated in high mol wt complexes as indicated by its elution in the void volume (Fig. 3, B and C; fraction 4–6). This transition from presumably monomeric CD59 to an association

with high mol wt complexes was time dependent and virtually complete by 3 h. Less CD59 was detectable in the cell lysates at later time points, reflecting the rapid loss of CD59 from the cell surface (Fig. 1). The kinase assay demonstrates the presence of kinases in immunoprecipitates by revealing phosphorylation of various proteins, one of which is the kinase itself. Analysis of immunoprecipitates of the gel filtration fractions in the kinase assay showed that the high mol wt complexes containing CD59 were associated with protein kinase activity (Fig. 3 D). In contrast, CD59 in the low mol wt fractions was devoid of kinase ac-

tivity. This was particularly notable at early time points, where the bulk of the CD59 was still low mol wt, but only the high mol wt CD59 coprecipitated kinases (Fig. 3, A and D; fractions at same time point).

### Effect of the Cholesterol-sequestering Agent Filipin

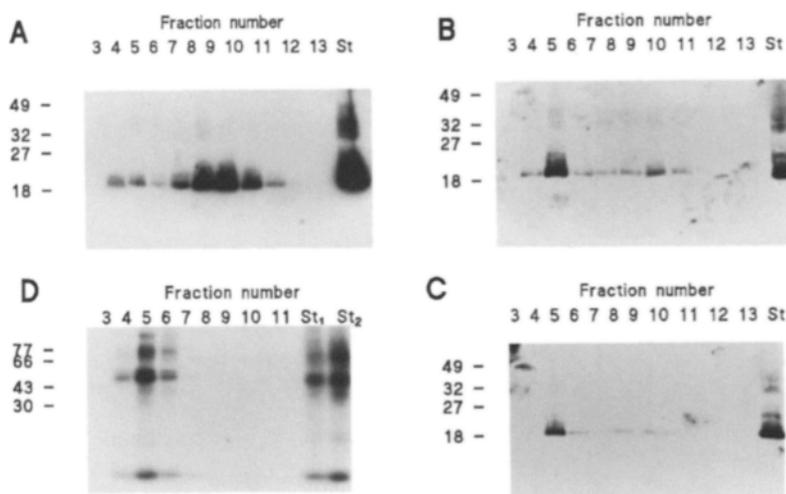
Cholesterol is reported to be important in the maintenance of GPI-rich clusters. We therefore examined the effects of the cholesterol sequestering agent filipin on the migration of incorporated CD59 into high mol wt complexes. U937 cells were incubated with CD59 and with filipin or DMSO as a control. After 1 h cells were lysed and lysates were fractionated on Sepharose 4B. Western blotting showed that filipin markedly inhibited the incorporation of CD59 into high mol wt complexes (Fig. 4, A and B). The *in vitro* kinase assay showed that incorporated CD59 also did not associate with kinase activity after filipin treatment of the cells (Fig. 4 C). The inability of incorporated CD59 to associate with high mol wt complexes after filipin treatment of the cells was caused by disruption by filipin of these high mol wt complexes as shown by the reduction of coprecipitation of kinase activity with endogenously expressed DAF (Fig. 4 D) and CD58 (not shown; both DAF and CD58 are GPI anchored and endogenously expressed by U937; van den Berg et al., 1994). To study the effect of filipin on clustered CD59, filipin was added 2 h after incorporation of CD59 and cells were incubated for another hour at 37°C. Control cells were incubated with carrier (DMSO) alone. Cells were stained for CD59 using mAb BRIC229 and GAM/IgG-TRITC and prepared for CLSM. TRITC-labeled secondary antibody had to be used since filipin itself was highly fluorescent at the wavelength required for FITC. Control cells stained for CD59 showed a clustered distribution of CD59 comparable to cells incubated with FITC-labeled CD59 (Fig. 5, A and B; cf. Fig. 2, D–F). Filipin had a marked effect on the distribution of CD59. The fluorescence became less clustered (Fig. 5, C and D) indicating that cholesterol sequestration had disturbed the GPI-anchored protein complexes. Similar results were obtained with FITC-labeled CD59 although images were clouded due to the filipin fluorescence (Fig. 5, E and F).

### Ca<sup>2+</sup> Signaling

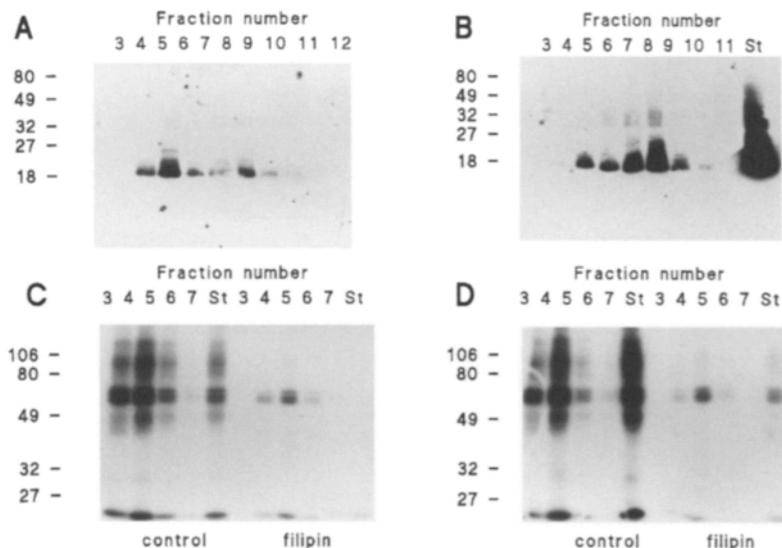
In the above described experiments we show a time-dependent association of incorporated CD59 with kinase activity hinting that acquisition of Ca<sup>2+</sup> signaling capacity by CD59 might also be time dependent. To test this, CD59 was incorporated into U937 and after 2 h, when, as shown by Western blotting all the CD59 was associated in high mol wt complexes, cells were loaded with Fura-2. After incubation with monoclonal anti-CD59, cross-linking using sheep anti-mouse IgG F(ab)<sub>2</sub> caused a rapid transient increase in [Ca<sup>2+</sup>] (Fig. 6). Control cells incubated with anti-CD59 (Fig. 6 A, dotted line) or cells incubated with CD59 but not anti-CD59 did not respond upon addition of cross-linking antibody (not shown). Both control cells and cells incubated with CD59 reacted upon cross-linking of endogenously expressed DAF (Fig. 6 B). As previously reported, newly incorporated CD59 did not transduce a signal upon cross-linking (not shown). It was not possible to assess the effects of filipin on the Ca<sup>2+</sup>-signaling because this agent induced membrane leakiness in U937 cells.

### Discussion

Perturbation of GPI-anchored molecules by cross-linking with immune complexes or antibodies causes activation in T cells and neutrophils, although natural ligands for the majority of these molecules have not yet been identified (Robinson, 1991; Morgan et al., 1993). The mediators of signal transduction are likely to be the tyrosine kinases that are tightly associated with GPI-anchored molecules in GPI-rich membrane clusters (Stefanova et al., 1991). Transfected GPI-anchored molecules also associate in GPI-rich clusters and transduce signals upon cross-linking, whereas transmembrane forms of these proteins are excluded from the complexes and do not signal upon cross-linking (Kroczek et al., 1986; Su et al., 1991; Shenoy-Scaria et al., 1992; Tiveron et al., 1994), indicating that the anchor is an essential prerequisite for signaling. However, it has been reported that exogenous GPI-anchored proteins, incorporated into the cell membrane through the anchor, were unable to signal upon cross-linking (Zhang et al., 1992; Morgan et al., 1993), implying that the anchor,



**Figure 3.** Western blots and kinase assay of cell lysates of U937 cells incubated with CD59 after gel filtration. U937 cells were incubated with 10 µg/ml CD59 for 15 min at 37°C. Cells were incubated in medium for various periods of time and lysed in 1% Brij58. Cell lysates were fractionated on Sepharose 4B and run on 15% SDS-PAGE under nonreducing conditions. After blotting onto nitrocellulose, blots were probed with anti-CD59. (A) *t* = 0 h; (B) *t* = 1 h; (C) *t* = 3 h incubation in medium. (D) Kinase assay of the fractions run in A using anti-CD59 as the immunoprecipitating Ab showing kinase association with CD59-containing high mol wt complexes (phosphorylated proteins in fractions 4–6) but not with monomeric CD59 (fractions 8–10). Numbers indicate fraction numbers off column. *St*, unfractionated cell lysate. (D) *St*<sub>1</sub> = *St* at 0 h; *St*<sub>2</sub> = *St* at 2 h.



**Figure 4.** Effect of filipin on incorporation of CD59. U937 cells were incubated with CD59 (5  $\mu\text{g/ml}$ ) in the presence of filipin (50  $\mu\text{g/ml}$ ) or DMSO (1%; control) for 1 h at 37°C. Cells were lysed in 1% Brij58 and lysates were fractionated on Sepharose 4B. Fractions were assayed by SDS-PAGE and Western blotting using anti-CD59 or in the kinase assay using anti-CD59 or anti-DAF as the immunoprecipitating Ab. Western blots: (A) control cells; (B) filipin treated, (C) kinase assay of anti-CD59 immunoprecipitates, (D) kinase assay of anti-DAF immunoprecipitates. Numbers indicate fraction numbers of column. *St*, unfractionated cell lysate.

though necessary, is not sufficient to confer signaling capacity. The above data suggests that the additional factor necessary for signaling might be association in the GPI-rich clusters. It appears that association of GPI-anchored molecules in kinase-containing clusters occurs during biosynthesis. Indeed, in some cell types, cluster formation has been shown to occur during transport through the Golgi system (Brown and Rose, 1992). It is thus possible that association in kinase-containing complexes occurs only during biosynthesis and exogenously added GPI-anchored molecules are irreversibly excluded and hence cannot signal.

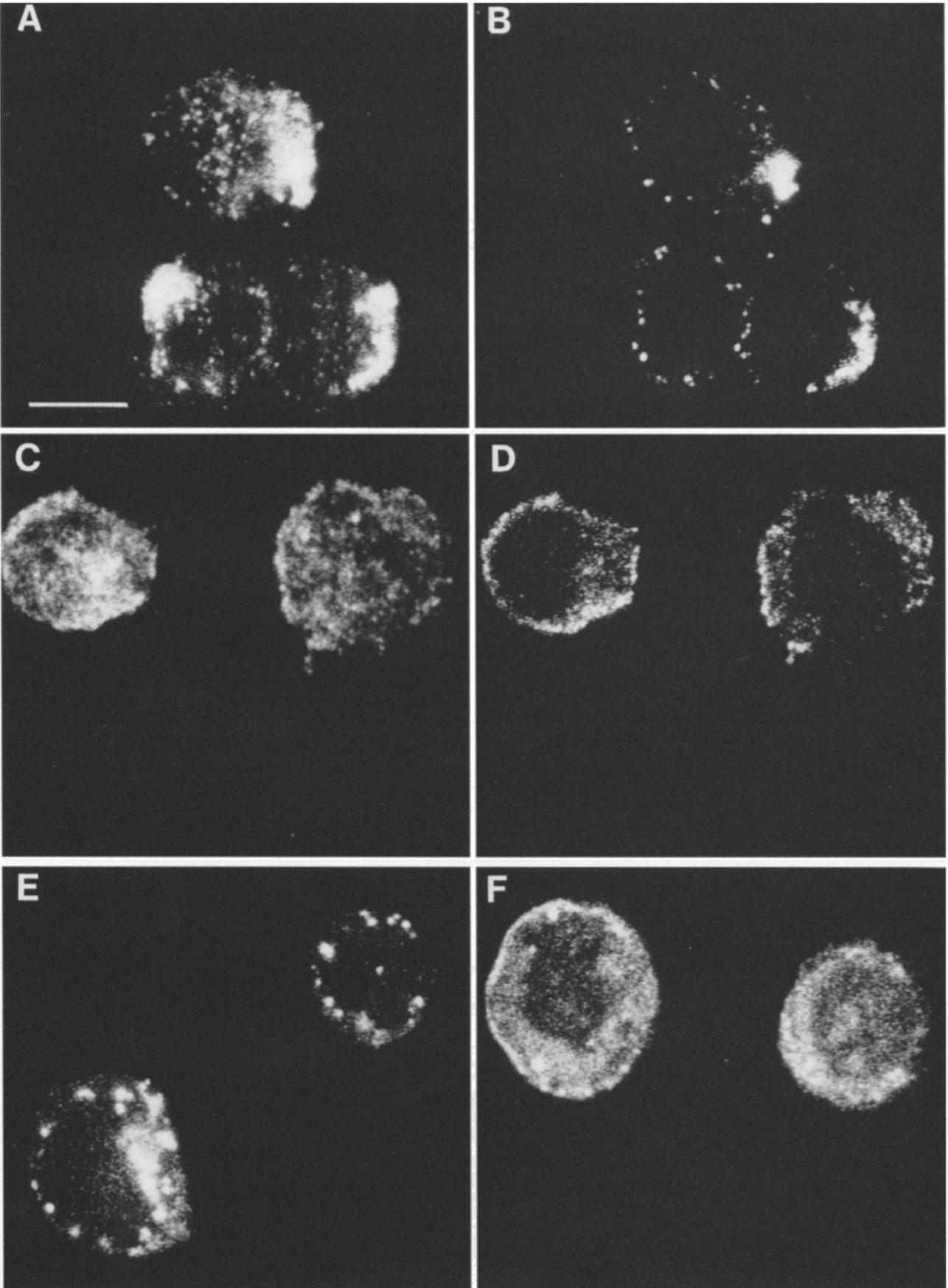
A second possibility is that association of exogenous GPI-anchored proteins with complexes is time dependent. Incubation times in these earlier studies were brief (Zhang et al., 1992: Thy1, 20 min at 37°C; Morgan et al., 1993: rat or sheep CD59, 30 min at 37°C), perhaps insufficient to allow association in complexes to occur. To investigate this possibility we studied the fate of incorporated GPI-anchored proteins. We incorporated erythrocyte CD59 in a CD59-negative U937 cell line and followed its distribution using various techniques. Using FACScan analysis, we found that CD59 rapidly incorporated in the cell membrane, maximal incorporation being achieved within 15 min at 37°C (Fig. 1 A). Both FACScan analysis and the use of  $^{125}\text{I}$ -labeled CD59 showed that incorporated CD59 was initially rapidly removed from the cell surface (50% within 2 h) and thereafter lost at a slower rate (Fig. 1, B and C). The equally rapid appearance of  $^{125}\text{I}$  in the supernatant suggested that loss of CD59 was primarily due to shedding but did not rule out an initial internalization event.

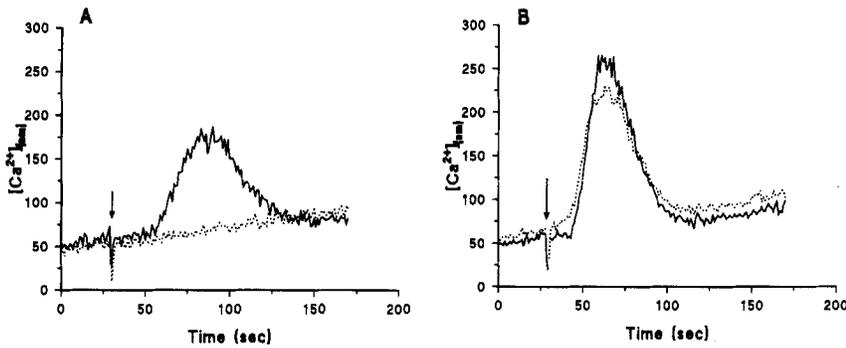
Although many cell types exhibit clustering of GPI-anchored proteins (Morgan et al., 1993; Tiveron et al., 1994; and our unpublished observations), it is apparent from the literature that this is not a ubiquitous phenome-

non (Fra et al., 1994; Parton et al., 1994). Moreover, controversy exists as to whether the observed clustering of GPI-anchored proteins on some cell types is an artifact induced during staining with fluorescent antibodies or during detergent solubilization of membranes. Mayor et al. (1994) argued that the clustered appearance of GPI-anchored molecules on cell lines of endothelial or fibroblast origin was an artifact induced during immunofluorescence staining after addition of second antibody and was not observed when directly labeled antibodies were used. In their studies, clustering could be prevented by rigorous fixation in 3% paraformaldehyde/0.5% glutaraldehyde. However, we have found a similar clustered staining pattern of the GPI-anchored molecules on U937 cells and also on neutrophils and K562 cells (data not shown) using either this fixation method or standard methods (1% paraformaldehyde) either at room temperature or on ice and using either labeled secondary antibody or directly labeled primary antibody (data not shown).

Proof that clustering on U937 cells was not an artifact of fixation or staining was provided by our demonstration that directly FITC-labeled CD59, which was diffusely distributed and of low mol wt (monomeric) immediately after incorporation (as judged by confocal microscopy or solubilization and gel filtration), became clustered and complexed during incubation at 37°C (Figs. 2 and 3). Confocal microscopy demonstrated that immediately after incorporation FITC-labeled CD59 was diffusely distributed (Fig. 2, A–C), unlike the clustered distribution of endogenous GPI-anchored proteins (DAF in this case) on these same cells (Fig. 2, G–I). Only upon further incubation at 37°C did the incorporated CD59 become clustered. Western blotting of cell lysates after gel filtration completely supported the confocal microscopy findings. Immediately af-

**Figure 5.** Effect of filipin on distribution of incorporated CD59: CLSM. (A–D) CD59 (10  $\mu\text{g/ml}$ ) was incorporated into U937 cells and 2 h after incubation at 37°C, cells were incubated with DMSO or filipin (50  $\mu\text{g/ml}$  final conc.) in DMSO. Cells were stained with mAb anti-CD59 and GAM/TRITC, fixed, and mounted. Control cells, incubated with DMSO: (A) extended focus, (B) cross-section of cells; filipin treated cells: (C) extended focus, (D) cross-section of cells. (E and F) FITC-labeled CD59 (10  $\mu\text{g/ml}$ ) was incorporated into U937 cells and cells were treated as above with filipin, fixed, and mounted. (E) control cells (cross-section) (F) filipin-treated cells (cross-section). Bar, 10  $\mu\text{m}$ .





**Figure 6.** Induction of  $\text{Ca}^{2+}$  flux by cross-linking incorporated CD59. U937 were incubated with CD59 (15  $\mu\text{g}/\text{ml}$ ) for 15 min at  $37^\circ\text{C}$  and incubated in medium for 2 h. Cells were loaded with Fura-2 and incubated with anti-CD59 (A) or anti-DAF (B). Cross-linking was induced using sheep anti-mouse IgG F(ab) $_2$ ; addition is marked with an arrow. Cells incubated with CD59, —; cells incubated with buffer, - - -.

ter incorporation, the CD59 eluted mainly as a monomer, with only a small amount in the void volume, indicating association in a high mol wt complex. However after 1 or 3 h incubation in medium, most or all of the CD59 eluted in the void volume, indicating that CD59 had migrated into detergent-resistant high mol wt complexes. The small amount of detergent in the CD59 preparation (<0.005% CHAPS) had no effect on the distribution of endogenous proteins, which remained in detergent-resistant complexes throughout the time course (not shown). Kinase assays showed that CD59 had also become associated with protein kinase activity, while the coimmunoprecipitation assay showed association with GPI-anchored DAF/CD55 but not with the transmembrane molecule MCP/CD46 (data not shown). Association with kinase activity was already prominent within 15 min but in all cases, as predicted from previous studies (Cinek and Horejsi, 1992), association was only observed with CD59 eluting in the void volume (Fig. 3, A and D). These studies, performed in the absence of any antibodies, effectively eliminate the possibility of experimental artifact and show concordance between imaging and biochemical techniques for demonstrating complexes.

The nature of the complexes remains an intriguing question. Others have suggested that caveolin represents an essential component of GPI-rich clusters in polarized cells (Lisanti et al., 1994). However, leukocytes and leukocyte-derived cell lines do not express caveolin (Fra et al., 1994; and our unpublished observation) and are not adherent, suggesting that the GPI-rich clusters we observe are structurally distinct from caveolae. In U937 cells, complexes were efficiently disrupted using the cholesterol-sequestering agent filipin, which has previously been shown to disrupt GPI-rich clusters in polarized cells (Rothberg et al., 1990). Filipin did not affect the ability of CD59 to incorporate into the cell membrane, but inhibited the association of CD59 with other molecules in high mol wt complexes as shown by gel filtration followed by Western blotting and kinase assay (Fig. 4) and also disrupted preformed clusters of CD59 as demonstrated by confocal microscopy (Fig. 5). These results confirm that cholesterol is important for the formation and stability of GPI-rich clusters.

The final aim of this study was to ascertain if incorporated CD59, after association with tyrosine kinases, also acquired signaling capacity. When cross-linking was induced immediately after incorporation, a time when most of the CD59 was still in the monomeric form, no calcium transient was observed, as previously reported (Morgan et

al., 1993). In contrast, when cross-linking of CD59 was induced 2 h after incorporation, when all the CD59 was in high mol wt kinase-containing complexes, a large calcium transient, equivalent to that obtained on cross-linking endogenous GPI-anchored proteins, was observed (Fig. 6). This clearly demonstrates that incorporated CD59, once associated with kinase activity, is able to transduce a signal. The results also show that GPI-rich clusters are not rigid entities produced exclusively during biosynthesis, but dynamic complexes which exogenously added GPI-anchored proteins can enter and acquire signaling capacity.

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