Cell Shape-dependent Control of Ca$^{2+}$ Influx and Cell Cycle Progression in Swiss 3T3 Fibroblasts*

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The ability of adherent cells such as fibroblasts to enter the cell cycle and progress to S phase is strictly dependent on the extent to which individual cells can attach to and spread on a substratum. Here we have used microengineered adhesive islands of 22 and 45 μm diameter surrounded by a nonadhesive substratum of polyhydroxyl methacrylate to accurately control the extent to which individual Swiss 3T3 fibroblasts may spread. The effect of cell shape on mitogen-evoked Ca$^{2+}$ signaling events that accompany entry into the cell cycle was investigated. In unrestricted cells, the mitogens bombesin and fetal calf serum evoked a typical biphasic change in the cytoplasmic free Ca$^{2+}$ concentration. However, when the spreading of individual cells was restricted, such that progression to S phase was substantially reduced, both bombesin and fetal calf serum caused a rapid transient rise in the cytoplasmic free Ca$^{2+}$ concentration but failed to elicit the normal sustained influx of Ca$^{2+}$ that follows Ca$^{2+}$ release. As expected, restricting cell spreading led to the loss of actin stress fibers and the formation of a ring of cortical actin. Restricting cell shape did not appear to influence mitogen-receptor interactions, nor did it influence the presence of focal adhesions. Because Ca$^{2+}$ signaling is an essential component of mitogen responses, these findings implicate Ca$^{2+}$ influx as a necessary component of cell shape-dependent control of the cell cycle.

Strict regulation of cell proliferation is necessary for controlled growth and development, tissue maintenance, and repair. Failure of the normal control of proliferation can lead to the development of tumors or hyperplasia. Mitogens trigger a series of intracellular signals including the elevation of intracellular Ca$^{2+}$, activation of protein kinase C, receptor tyrosine kinases, and in particular the Ras-regulated, Raf, MAP6 kinase/ERK kinase (MEK), ERK1/2 kinase cascade (1–4). Mitogen-evoked Ca$^{2+}$ responses are essential for cell cycle entry and progression through G1 (5–7). However, growth factors acting alone cannot stimulate proliferation. In addition, a cell must receive the correct environmental cues. These are derived from cell-substrate adhesion (8, 9), cell shape (10–12), and principally, the absence of cell-cell contact (13). Recently, it has come to light that cell-cell contact can exert either pro- or anti-proliferative responses depending upon the activity of Rho kinase (14). Consequently, growth factors will not stimulate proliferation in nonadhered cells, and cell adhesion alone is insufficient to trigger proliferation. Growth factors will not stimulate adherent cells to proliferate in culture unless the cells are allowed to spread.

Transient stimulation by a mitogen is not sufficient to trigger proliferation, and the presence of mitogen is required for many hours until the cells progress past the restriction point in G1 (15, 16). Thereafter, the cell is committed to divide, even if the mitogen is withdrawn, or the cell is no longer in contact with a substrate (17–19). This restriction point is characterized by hyperphosphorylation of the retinoblastoma protein and subsequent activation of the E2F transcription factor (20, 21). Activity of ERK1/2 is critical in reaching the restriction point because it regulates the expression of cyclin D3 that in turn regulates the activity of the cyclin-dependent kinases cdk4 and cdk6 (1, 19, 22). These cdks along with cdk2 (23) are responsible for phosphorylating the retinoblastoma protein (1, 24), thereby relieving its inhibition of transcription. Mitogens evoke a biphasic increase in ERK1/2 activity involving a large initial increase in activity followed by a degree of activity sustained at a lower level (2, 22, 23). If the sustained activity is either too high or too low, the cells will arrest in G1 (1, 25, 26). Without cell adhesion and integrin engagement, the critical activity of ERK1/2 is too transient to trigger progression past the restriction point (27, 28).

The role of cell shape in cell cycle progression is also critical (29). Studies using adhesive islands of varying diameters that strictly control cell spreading revealed that the degree of spreading dictates the proportion of cells that proliferate (10–12). When cell spreading is severely restricted, the actin...
cytoskeleton is rearranged, revealing the formation of cortical actin and loss of stress fibers (30). Disruption of cell shape with agents that eliminate actin stress fibers also inhibits proliferation (17, 29, 31). Rho-dependent tension in the stress fibers is required for the all-important, maintained activity of ERK1/2 that leads to expression of cyclin D1. Interestingly, inhibition of Rho kinase reveals an alternative, stress fiber-independent route to cyclin D1 expression. This Rho kinase-suppressed pathway is mediated by Rac1 and cdc42 (27), but its activation still requires the presence of a mitogen as well as integrin engagement. In addition, RhoA is reported to regulate p27kip1 degradation through its effector, mDia and expression of Skp2 (32). However, activation of the Rho-mDia/Rho kinase-Skp2-p27 pathway in rounded cells does not fully mimic the shape-dependent signal for G1 progression (32). Consequently additional signaling pathways must be involved in transducing the downstream effects of cell shape to the cell cycle machinery.

Ca²⁺ signaling is also affected by the state of the actin cytoskeleton. Pharmacological agents that affect the cytoskeleton and hence cell shape-dependent cell cycle progression also affect Ca²⁺ signaling (33–36). Consequently Ca²⁺ signaling may form a link between cell shape and cell cycle progression. To investigate this possibility, we made use of adhesive palladium islands (11) to control the extent of cell spreading. Using Swiss 3T3 cells synchronized in G0, we investigated the effects of cell shape on the bombesin- and FCS-evoked Ca²⁺ responses and on the ability of the cells to progress through G1. We also examined how cell shape affected the cytoskeleton and the presence of focal adhesions. Our key finding was that restricting cell shape dramatically affects the profile of the bombesin- and FCS-evoked [Ca²⁺]ₗ responses. Ca²⁺ release appears to be unaffected, but Ca²⁺ influx is abolished. The ramifications for cell cycle progression are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Swiss 3T3 fibroblasts were a kind gift from Prof. E. Rozengurt (Imperial Cancer Research Fund Laboratories, London, UK). Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), and 0.05% trypsin in 0.02% EDTA were purchased from Invitrogen. Penicillin, streptomycin, bombesin, bovine serum albumin, lubrol, polyhydroxyethyl methacrylate (polyHEMA), and primary antibodies raised to α-actinin or talin were supplied by Sigma. Triton X-100, paraformaldehyde, ammonium chloride, glucose, sodium citrate, sodium bicarbonate, and HEPES were obtained from VWR International Ltd. (Leicestershire, UK). Anti-mouse immunoglobulin FITC conjugates were purchased from Dako Ltd. (Ely, UK). VectaShield was supplied by Vecta Laboratories Ltd. (Peterborough, UK). Fura-2 AM was purchased from Molecular Probes (via Cambridge Bioscience Ltd., Cambridge, UK). Propidium iodide and ionomycin were obtained from Molecular Probes (via Cambridge Bioscience Ltd., Cambridge, UK). Anti-mouse immunoglobulin FITC conjugates were purchased from Dako Ltd. (Ely, UK). VectaShield was supplied by Vecta Laboratories Ltd. (Peterborough, UK). Fura-2 AM was purchased from Molecular Probes (via Cambridge Bioscience Ltd., Cambridge, UK). Propidium iodide and ionomycin were obtained from Calbiochem (Nottingham, UK).

**Cell Culture**—Swiss 3T3 cells were cultured in DMEM supplemented with 10% FCS, 44 mM sodium bicarbonate, 25 mM glucose, penicillin-G (35 units/ml), and streptomycin (80 milli-units/ml). The cells were incubated at 37 °C in an atmosphere of 10% CO₂ and 95% air. Swiss 3T3 cells were subcultured at ~70% confluence three times/week. The cells were incubated with 0.05% trypsin in 0.02% EDTA for 4 min at 37 °C. The cells were then added to 10 ml of 10% FCS-DMEM and centrifuged at 140 × g for 5 min and split at a ratio of 1:4 in 10% FCS-DMEM.

**Preparation of Adhesive Palladium Islands**—Clean glass coverslips were coated with polyHEMA (37). Briefly, a 1% (w/v) solution of polyHEMA in 95% ethanol was prepared and was evenly coated on the coverslips. The polyHEMA-coated coverslips were air-dried at room temperature for 90 min and then at 60 °C for a further 90 min. Palladium was then deposited onto the surface of the polyHEMA using an Edwards 12E6 vacuum coating unit. The palladium was evaporated through photo-lithographically etched copper foils containing perforations of either 45 or 22 diameter μm to form large or small islands set 200 μm apart (11). Prior to use in experiments, the coverslips were sterilized by UV irradiation for 20 min and were then stored in a desiccator cabinet until required.

**Maintenance of Fibroblasts on Palladium Islands**—Confluent and quiescent monolayers of cells 5 days old were washed with 10 ml of versene (136.8 mM NaCl, 2.68 mM KCl, 6.21 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 0.54 mM EDTA). The cells were incubated with 0.05% trypsin in 0.02% EDTA for 4 min at 37 °C. The cell pellet was resuspended in 10% FCS-DMEM, and 2-ml aliquots containing cells were seeded at a density of 2 × 10⁴ cells/ml for large islands and 8 × 10⁴ cells/ml for small islands, resulting in single cell occupancy of islands (38). The cells were incubated in a humidified atmosphere for 1 h and were washed three times with 1 ml of 10% FCS-DMEM to remove remaining unattached cells. These coverslips were transferred to a fresh dish and incubated for a further 2 h in 2 ml of 10% FCS-DMEM to enable attachment and spreading. The coverslips were subsequently washed three times with 1 ml of serum-free DMEM and were then incubated in 2 ml of serum-free DMEM for 16 h, so that the cells entered quiescence.

**Visualization of Actin Cytoskeleton**—The cells were washed in PBS (136.8 mM NaCl, 2.68 mM KCl, 6.21 mM Na₂HPO₄, 1.47 mM KH₂PO₄) and fixed in 2% paraformaldehyde in PBS for 30 min and then rinsed in cell lysis buffer (10 mM NaH₂PO₄, 100 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 μM 2,0 glycerol) for 30 min. The cells were permeabilized in cell lysis buffer containing 5 mg/ml bovine serum albumin, and 1% Lubrol (w/v) for 30 min and subsequently incubated in FITC phalloidin (2.5 μg/ml) for 60 min at room temperature in the dark. They were rinsed three times with cell lysis buffer to remove excess phalloidin as previously described (39).

**Focal Adhesion Staining**—The cells were washed in PBS for 10 min and then fixed in 4% paraformaldehyde in PBS for 30 min. Excess paraformaldehyde was then quenched in ammonium chloride (50 mM) for 10 min before permeabilization in 0.1% Triton X-100 for 10 min. Nonspecific binding was blocked by incubating in 0.1% bovine serum albumin in PBS for 10 min before washing with PBS for 10 min. The cells were incubated with primary antibodies (3 μg/ml) raised to α-actinin or talin for 5 h. The cells were then washed three times with PBS and incubated with secondary antibodies, 3 μg/ml anti-mouse IgG FITC-conjugated antibody overnight at 4 °C. Finally, coverslips were washed five times in PBS for 10 min to remove unbound antibodies (40).

**Assessment of the Cell Cycle by Flow Cytometry**—The progression of cells through the cell cycle in response to fetal calf
serum stimulation was assessed using the propidium iodide hypotonic citrate method (41). Quiescent cells were maintained on adhesive islands or unrestricted on glass coverslips as described above and were incubated either in serum-free DMEM or in DMEM-10% FCS for 24 h at 37 °C. The cells were washed twice with ice-cold PBS, pH 7.4, and subsequently incubated in 0.03% Triton X-100 in 4 mM sodium citrate, pH 7.6, containing 0.2 μg/ml RNase and 10 μg/ml propidium iodide for 30 min at 4 °C. The cells were recovered by gentle pipetting and were analyzed by flow cytometry using a 15-milliwatt FACScan flow cytometer (Becton Dickinson) with a 488-nm line from an air-cooled argon ion laser. Data analysis was performed using the WinMDI (version 2.8) software package. In each island study, the data were collected from at least three separate experiments involving at least five individual samples of 2,000 cells.

**Measurement of Intracellular Calcium**—The intracellular calcium concentration was measured by monitoring the fluorescence emission of cells loaded with Fura-2 AM (43). The cells were immersed in HEPES-buffered saline (145 mM NaCl, 5 mM KCl, 1 mM NaH₂PO₄, 10 mM HEPES, 10 mM glucose, 1 mM MgSO₄) with either 1 mM CaCl₂ or 1 mM EGTA. After 16 h in serum-free conditions, the cells were loaded with 2 μM Fura-2 AM at 37 °C for 45 min in HEPES-buffered saline also containing 0.1% bovine serum albumin, 0.0125% pluronic F127, and 200 μM sulfinpyrazone. After loading, the coverslips were placed in a purpose built thermostatted chamber. Changes in fluorescence were monitored with a PTI Deltascan imaging system coupled to a Nikon Diaphot inverted microscope and Photon Science Ltd. (Robertsbridge, UK) Extended ISIS camera (42, 43). The data were analyzed using PTI ImageMaster software. Calibrations and manganese quench were performed using standard protocols (44–46).

**Confocal Microscopy**—FITC-phalloidin and immunofluorescently labeled cells were imaged using a Leica SP2 AOBS confocal microscope.

**RESULTS**

**The Effect of Restricting Spreading on Cellular Morphology**—Bright field images of Swiss 3T3 cells plated onto large and small adhesive islands or onto a nonrestrictive palladium-coated glass surface are shown in Fig. 1. The cells in the bottom panels (glass/palladium) have the typical spread morphology of fibroblasts in culture. The middle panels show the appearance of cells plated on microarrays of 45-μm diameter islands (five of the nine islands are occupied in the middle left panel). The top panels reveal the appearance of cells on the small, 22-μm diameter adhesive islands. The degree of spreading is clearly restricted, and these cells have a dome-like morphology (11, 12). The main difference between unrestricted cells and those on large islands is that the unrestricted cells adopt a range of polygonal type shapes, whereas on the large islands the cells are uniformly circular when viewed from the top down. Importantly, the overall degree of spreading between these two conditions is quite similar, especially when they are compared with the cells on the small islands. Membrane ruffling is a feature of unrestricted cells, and a small degree of ruffling can also be observed with the cells on the large islands but not with the cells on the small islands.

**FIGURE 1.** Bright field images of Swiss 3T3 fibroblasts. The bright field images are of Swiss 3T3 cell plated onto palladium-coated islands surrounded by nonadhesive polyHEMA and maintained in the absence of serum for 24 h. Small (22 μm diameter) and large (45 μm diameter) islands are shown along with cells with no restriction on spreading. Scale bar, 50 μm.

**FIGURE 2.** Restriction of cell spreading prevents cell cycle progression. DNA content of Swiss 3T3 cells was measured by propidium iodide staining and flow cytometry. Confluent-quiescent Swiss 3T3 cells were plated onto either small or large adhesive islands or nonrestrictive glass coverslips and maintained in DMEM for 16 h in the absence of FCS. The cells were then incubated in the presence or absence of 10% FCS for a further 24 h. Peaks correspond to either normal DNA content (n) or twice normal DNA content (2n).
Restriction of Cell Spreading Prevents S Phase Progression—DNA content was measured by flow cytometry and propidium iodide staining following mitogen stimulation with 10% FCS. The bottom right panel of Fig. 2 shows the effects of adding serum to cells on a nonrestrictive surface. The bottom left panel shows the serum-free control. Typically, 70% of these cells progressed to G2/M after 24 h when stimulated with FCS as demonstrated by the increase in the peak reporting 2 DNA content. Again, ~70% of the cells on large islands also progressed to G2/M when stimulated with 10% FCS, whereas only ~25% of cells on small islands progressed to G2/M, with the most of the cells arrested in G0/G1. These data are in close agreement with the landmark experiments conducted by Folkman (10) and Ireland and co-workers (11, 12).

Manipulation of Cell Shape Alters Ca2+ Signaling—Because elevations of [Ca2+]i are known to be essential for G1 progression and cell proliferation, as well as being an integral part of mitogen signaling, we examined the effects of plating cells on restrictive islands on mitogen-evoked [Ca2+]i responses. In control experiments, with cells plated on glass or palladium, a typical biphasic elevation of [Ca2+]i ([Ca2+]i peak followed by elevated plateau) was observed when the cells were treated with either 10 nM bombesin (Fig. 3c) or 10% FCS (Table 1). When extracellular Ca++ was removed, both bombesin and FCS evoked a transient elevation of [Ca2+]i that rapidly returned to baseline and was not followed by a plateau of elevated [Ca2+]i (Fig. 3e and Table 1). These findings are consistent with Ca2+ influx being required for the plateau phase of the Ca2+ response. When the cells were placed on the large adhesive islands, mitogen stimulation resulted in [Ca2+]i responses that were similar to those observed in cells on the nonrestrictive surfaces (Fig. 3, compare c with a and 3b and Table 1). In marked contrast, however, when the cells were plated on the small adhesive islands, the mitogen response consisted only of the initial transient spike in [Ca2+]i, and no sustained element of elevated plateau occurred (Fig. 3d and Table 1). These data indicate that mitogens evoke a release of Ca2+ from intracellular stores but not an influx of Ca2+ from the extracellular environment when Swiss 3T3 cells are plated on a restrictive surface that prevents cell spreading. The responses are essentially the same as those seen in the presence of the Ca2+ chelator, EGTA (Fig. 3). These data are summarized in Table 1.

To further substantiate that Ca2+ influx is affected when cells are prevented from spreading, we made use of the ability of extracellular Mn2+ to act as a surrogate for Ca2+ and thereby quench the fluorescence of intracellular Fura-2 (45) (Fig. 4). When unrestricted cells plated on either glass or palladium were stimulated with bombesin in the presence of extracellular Mn2+, both the 340- and 360-nm signals were quenched (Fig. 4, a and b). The same occurred when cells plated on large islands were stimulated with bombesin (Fig. 4).

**Table 1**

| Substrate                  | Response          | Fetal calf serum | Bombesin |
|----------------------------|-------------------|------------------|----------|
| Glass unrestricted         | Peak-basal        | 331.1 ± 33.2 (n = 18) | 402.3 ± 33.9 (n = 43) |
| Glass unrestricted + EGTA  | Plateau-basal     | 40.3 ± 4.6 (n = 18)  | 53.1 ± 8.1 (n = 43)  |
| Palladium unrestricted     | Peak-basal        | 574.8 ± 41.4 (n = 12) | 423.3 ± 46.5 (n = 15) |
| Large island               | Peak-basal        | 13 ± 9.4 (n = 12)* | -26.7 ± 4.3 (n = 15)* |
| Small island               | Peak-basal        | ND                | 331.8 ± 55.6 (n = 23) |
|                            | Plateau-basal     | ND                | 33.3 ± 3.5 (n = 23)  |

* Significant difference from control with *p* < 0.05.

**FIGURE 3:** Restriction of cell spreading alters bombesin-induced Ca2+ signaling. Quiescent Swiss 3T3 cells were stimulated with 10 nM bombesin in the presence of either 1 mM extracellular Ca2+ (a–d) or 1 mM EGTA (e). Responses are shown from unrestricted cells on glass (a and e) and on palladium (b) and from restricted cells on either large (c) or small (d) adhesive islands. The presence of bombesin is indicated by the solid bar labeled Bom. The mean data are presented in Table 1.
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Figure 4. Restriction of cell spreading inhibits bombesin-evoked Mn\(^{2+}\) quench of intracellular Fura-2. Quiescent Swiss 3T3 cells were stimulated with 10 nM bombesin in the presence of 1 mM Ca\(^{2+}\) and 1 mM Mn\(^{2+}\). The 340- and 360-nm signals of Fura-2 are shown as indicated. The figure shows responses from unrestricted cells on glass (a), palladium (b), large islands (c), and small islands (d). The presence of Mn\(^{2+}\) and bombesin (Bom) are indicated by solid bars. Panel e shows the mean changes in rate of quench of the 360-nm signal resulting from the addition of bombesin. The traces are representative of between 10 and 19 similar experiments. There was no significant difference between the delta quench rates on glass palladium and large islands (analysis of variance \(p > 0.1\)). * The delta quench rate on small islands was significantly lower (\(p < 0.05\), Student's t test) than large island, palladium, or glass controls.

4c), but not when cells plated on the small restrictive islands (Fig. 4d) were examined. The mean change in rate of quench of the 360-nm signal upon the addition of 10 nM bombesin for each of the plating conditions is shown Fig. 4e. The data presented in Figs. 3 and 4 demonstrate that restricting cell spreading selectively affects mitogen-evoked Ca\(^{2+}\) influx.

When we compared the peak height of the initial Ca\(^{2+}\) spikes, there was no statistically significant change in the peak height for both bombesin- and FCS-evoked responses whether or not the cells were plated on glass, palladium, or large or small restrictive islands (Table 1). This indicates that the mitogen-receptor interactions are not affected by cell shape. To confirm that changing ligand-receptor interactions would affect the peak [Ca\(^{2+}\)]\(_{c}\) responses, we generated a concentration-response curve to bombesin in unrestricted cells (Fig. 5). Bombesin at 10 nM was maximal for promoting a [Ca\(^{2+}\)]\(_{c}\) response. Reducing the concentration of bombesin caused a clear reduction in the peak height of the release response (Fig. 5). These data indicate that reducing mitogen-receptor interactions is reflected in a change in the peak height of the initial response. Consequently, changes in mitogen-receptor interactions are unlikely to be responsible for the selective removal of the Ca\(^{2+}\) influx response as seen with cells on the small islands.

The Effect of Restricting Cell Spreading on the Cytoskeleton—Manipulating the cytoskeleton can affect both Ca\(^{2+}\) influx and cell proliferation. Therefore, the effect that plating the cells onto islands had on the cytoskeleton was determined (Fig. 6). In the first instance we looked at actin using FITC-phalloidin and found that even when cells were grown on the large islands, there was disruption of the normal actin stress fibers that are characteristic of spread cells (Fig. 6, compare a and b with c). Stress fibers are almost completely absent, and there is an increase in cortical actin. When cells were plated onto the small islands, the stress fibers disappeared, and a clear ring of cortical actin was present (Fig. 6d).

Because adhesion is critical to cell spreading and focal adhesions interact with actin filaments, we examined how the distribution of focal adhesions was affected by our plating protocols. Immunofluorescence of \(\alpha\)-actinin (Fig. 7, a, c, and e) and talin (Fig. 7, b, d, and f) indicated the continued presence of focal adhesions when the cells were plated on either large or small adhesive islands. The distribution of focal adhesions was altered, however, with a proportion of focal adhesions appearing in a peripheral ring (Fig. 7, c–f) reflecting the change in actin distribution. Importantly, there were no marked differences in the extent or distribution of focal adhesions when comparing cells on large or small adhesive islands. Furthermore, for cells on small islands, focal adhesions were present throughout the plane of contact with the substratum and were not just located in a peripheral ring (Fig. 7, e and f). Manipulating cell spreading had no visible effect on the distribution of microtubules when examined by immunofluorescence (data not shown). Plating the cells on adhesive islands clearly brings about changes in the cytoskeleton. The main effect appears to be on actin; focal adhesions remain visible, and their altered distribution largely appears to reflect the changes in actin.
Although cell shape is recognized as one of the cornerstones of cell cycle regulation (12, 29, 47), the relationship between cell shape and G1 progression is still not fully understood. Rho signaling is clearly important (27, 32), and downstream effectors and resulting changes in the status of the cytoskeleton can regulate transcriptional events (48–50). However, it is clear that other signaling processes are involved. Here, we present data that reveals Ca\(^{2+}\) as an additional factor in shape-dependent regulation of proliferation. In this study, adhesive islands of varying size have been used to investigate the relationship between cell spreading and cell cycle progression. It is confirmed that restricting cell spreading prevents the normal entry into S phase that occurs in response to mitogen stimulation (10, 12). Importantly, it is shown that when cells were prevented from spreading, the typical biphasic \([Ca^{2+}]_c\) response induced by both serum and bombesin was altered with the loss of Ca\(^{2+}\) influx. Ca\(^{2+}\) influx is widely acknowledged to be essential for G1 progression (5, 6, 51), and therefore its loss would be a critical event. Evidence for the disappearance of Ca\(^{2+}\) influx came from observations that the plateau phase of the mitogen-evoked \([Ca^{2+}]_c\) was lost, and mitogens only evoked a transient release of Ca\(^{2+}\) from intracellular stores when cells were maintained on small islands. Likewise, the Mn\(^{2+}\) quench of intracellular Fura-2 was inhibited when the Swiss 3T3 cells on small islands were stimulated in the presence of Mn\(^{2+}\) (Mn\(^{2+}\) is well established as surrogate for Ca\(^{2+}\) influx studies (45, 46, 52)).

In light of these findings, one possible explanation was that cell shape might be giving rise to these differences through an effect on mitogen-receptor interactions. However, this possibility is not supported by the observation that the initial phase of the Ca\(^{2+}\) response, the release of Ca\(^{2+}\) from intracellular stores, was not affected by cell shape. A concentration-response curve to bombesin in unrestricted cells also showed that when the bombesin concentration was reduced, the peak height of Ca\(^{2+}\) release also decreased. Because the release of Ca\(^{2+}\) from intracellular stores was unaffected by island size and therefore cell spreading, these data strongly support the hypothesis that there is no shape-dependent affect on the mitogen-receptor relationship. Consequently, we propose that downstream events that couple receptor activation to Ca\(^{2+}\) influx must be regulated by cell shape. Because FCS is likely to work via growth factor receptor-mediated activation of phospholipase C\(\gamma\) (53, 54) and bombesin via G-protein-coupled receptor-mediated activation of phospholipase C\(\beta\) (53, 54), the effects of cell shape on Ca\(^{2+}\) influx appear to be independent of signalling.
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the transduction pathway by which the response was initially activated.

Recently it has been reported that Ca\textsuperscript{2+} release and subsequent activation of NF-\kappa B were essential for G\textsubscript{0}-G\textsubscript{1} transition in Swiss 3T3 cells (55). Indeed, multiple steps in G\textsubscript{0}-G\textsubscript{1}-S phase progression are influenced by Ca\textsuperscript{2+} (5, 7, 56–59). Critical events in G\textsubscript{1} such as cAMP response element-binding protein activation, immediate early gene lifetime, MAP kinase phosphatase-1, protein kinase C, nuclear factor of activated T cells, and S100/P53 in addition to NF-\kappa B are all affected by Ca\textsuperscript{2+}. Roles for Ca\textsuperscript{2+} influx have been proposed for both early and late in G\textsubscript{1} (7). One attractive scenario is that Ca\textsuperscript{2+} influx acting through calmodulin kinase regulates the biphasic activity of MAP kinase (60). G\textsubscript{1} progression requires an initial peak of MAP kinase activity upon mitogen stimulation that is followed by a lower sustained level of MAP kinase activity (1, 2, 22). If the kinase activity is too high, then G\textsubscript{1} progression may arrest through continued activation of cyclin-dependent kinase inhibitors p21/p27 and targeting of cyclin D\textsubscript{1} to the cytoplasm (1, 25, 26). In addition, we have reported previously that SK&F 96365, an inhibitor of Ca\textsuperscript{2+} influx, inhibits G\textsubscript{1} progression in Swiss 3T3 cells (60). SK&F 96365 also brings about a change in expression of 29 genes at the mRNA level and 22 genes at the level of proteins. Some of the genes identified, notably PTEN, tristetrapolin, and inhibin/activin, may be additional targets for Ca\textsuperscript{2+} regulation of the cell cycle. Although there may be many facets to the control of cell cycle by Ca\textsuperscript{2+}, our key finding is to link cell shape-dependent control of the cell cycle specifically to Ca\textsuperscript{2+} influx.

Changes in cell adhesion have also been reported when cell spreading is prevented. A ring of focal contacts is associated with the cortical ring of microfilaments (11). Using immuno-fluorescence, it was found that cells on large and small adhesive islands still form focal contacts as indicated by hot spots of \(\alpha\)-actinin and talin. Unlike in the earlier studies (11) we did not see evidence for a tight ring of focal contacts, but a proportion of focal contacts were certainly orientated in a peripheral ring. However, many focal contacts were also found across the whole plane of contact even when the cells are on small islands. We cannot comment on their turnover (11, 30), but nonetheless, gross disruption of focal adhesions is not likely to have caused either the failure to progress to S phase or the loss of Ca\textsuperscript{2+} influx. In any case, cell spreading rather than the surface area of adhesion is considered to be the main determinant of whether a cell progresses through G\textsubscript{1} (61).

One of the most striking changes that occurs when cells are grown on small adhesive islands is in the actin cytoskeleton. Generally, the changes in the cytoskeleton observed here are in agreement with earlier studies by Ireland et al. (11, 30). Restricting cell shape changes the distribution of actin; the typical stress fibers seen in spread cells are lost and replaced with a cortical ring of actin. However, it should be noted that dramatic changes in the actin cytoskeleton also occur in cells grown on large islands, yet these cells demonstrate Ca\textsuperscript{2+} influx and progression to S phase. Consequently modification of the actin architecture in this scenario is not sufficient to prevent progression to S phase. Interestingly, a recent report indicates that arrest of fibroblasts in G\textsubscript{1} in response to actin inhibitors is mediated via the retinoblastoma protein without a change in p53 status or even a significant rearrangement of the actin (62).

In other studies, Rho kinase activity is implicated in cell shape-dependent control of the cell cycle, with Rac/cdc42 pathway leading to cyclin D\textsubscript{1} expression without actin polymerization and tension (27, 47). Microtubules are another major component of the cytoskeleton, and their disruption can lead to G\textsubscript{1} arrest through p53 (63). Growing cells on restrictive islands, however, did not induce any detectable changes in the integrity of the microtubule networks.

In addition to affecting cell shape, the cytoskeleton, and cell proliferation, agents such as cytochalasin D and jasplakinolide are also associated with the inhibition of Ca\textsuperscript{2+} influx (33–36). The former depolymerizes F-actin, whereas the latter stabilizes actin microfilaments. In some systems cytochalasin D does not necessarily inhibit Ca\textsuperscript{2+} influx (33, 64), and its actions appear to be time- and channel-dependent (35, 65, 66). However, there appears to be a consensus that jasplakinolide inhibits Ca\textsuperscript{2+} influx (35, 64, 66), and interestingly, this agent induces the formation of a ring of cortical actin reminiscent of that seen with cells on small islands (33). These agents cause considerable disruption to the normal cell architecture and in some instances are used at concentrations where selectivity may be an issue. Our data unequivocally show that a change in cell shape, in otherwise normal untreated cells, is sufficient to affect Ca\textsuperscript{2+} entry. How it does so remains unknown, but the data from large islands suggest that the relationship between the actin cytoskeleton and Ca\textsuperscript{2+} influx is not necessarily deterministic and warrants further investigation.

The nature of the influx pathway needs to be defined. Capacitative Ca\textsuperscript{2+} entry now appears to work via STIM1 regulation of the Ca\textsuperscript{2+} channel Orai1 (67–72). However, TRPC channels or...
arachidonic acid-activated channels could also mediate the cell shape-sensitive Ca\(^{2+}\) influx seen here. In addition to Orai1, STIM1 has been suggested regulate TRPC1 and the arachidonic acid-activated Ca\(^{2+}\) channel (73–75). Hence, both capacitative Ca\(^{2+}\) entry and noncapacitative Ca\(^{2+}\) entry could be cell shape-sensitive and elements involved in the activation of these channels such as translocation of STIM1 or cytosolic phospholipase A\(_2\) could be affected by the presence of cortical actin or other changes in cell architecture.

In conclusion, we demonstrate that Ca\(^{2+}\) influx, an essential component of mitogen-activated G\(_1\) progression, is lost when cell spreading is prevented in Swiss 3T3 cells. This finding implicates Ca\(^{2+}\) influx as a necessary component of cell shape-dependent control of the cell cycle.

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