

The role of transient ERK2 signals in fibronectin- and insulin-mediated DNA synthesis

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SUMMARY

Both the extracellular matrix and growth factors jointly regulate cell cycle progression via a complex network of signaling pathways. Applying quantitative assays and analysis, we demonstrate here that concurrent stimulation of Chinese hamster ovary (CHO) cells with fibronectin (Fn) and insulin elicits a DNA synthesis response that reveals a synergy far more complex than a simple additive enhancement of response magnitude. CHO cell adhesion to higher Fn density shifts the sensitivity of the DNA synthesis response to insulin concentration from smoothly graded to sharply 'switch-like' and dramatically decreases the insulin concentration required for half-maximal response by about 1000-fold. Conversely, treatment with insulin has a milder and less complex effect on the response to varying Fn concentrations. Governing this DNA synthesis response is a common requirement for a transient, cell area-independent extracellular signal-regulated kinase 2 (ERK2) signal. Moreover, we show that the time-integrated value of

this 'pulse' signal provides an appropriate metric for quantifying the dependence of DNA synthesis on the degree of ERK2 activation. Indeed, in the absence of insulin, the adhesion-mediated response is linearly proportional to ERK2 activation over a broad range of stimulatory Fn and MEK inhibitor amounts. However, in the presence of both Fn and insulin, total integrated ERK2 activity (the sum of Fn- and insulin-mediated signals) no longer serves as a predictor of DNA synthesis, demonstrating that the signaling crosstalk underlying response synergism does not converge at ERK2 activation. Instead, adhesion to higher Fn density enhances insulin stimulation of DNA synthesis, not by increasing insulin-mediated ERK2 activation, but via parallel elevation of at least one other insulin-mediated signal such as IRS-1 phosphorylation.

Key words: Adhesion, Cell cycle, DNA synthesis, Fibronectin, Insulin, ERK2

INTRODUCTION

Adhesion to extracellular matrix (ECM) proteins initiates a wide range of signaling events (Clark and Brugge, 1995; Edwards and Streuli, 1999; Giancotti and Ruoslahti, 1999; Schwartz et al., 1995), many of which are also triggered or affected by growth factor (GF) stimulation (Schwartz and Baron, 1999; van der Geer and Hunter, 1994). Moreover, this interconnectivity between ECM- and GF-mediated signals extends to adhesion-mediated structural responses such as the formation of cortical actin structures (Aplin and Juliano, 1999) or focal adhesion complexes, in which not only clustered integrin adhesion receptors, but also growth factor receptors, are found (Miyamoto et al., 1996; Plopper et al., 1995). This high degree of signaling crosstalk gives rise to joint regulation of numerous cell responses by the ECM and soluble factors (Giancotti and Ruoslahti, 1999; Sastry and Horwitz, 1996). A major example of such coregulation occurs in cell cycle progression, in which most normal cells require adhesion to proliferate even in the presence of soluble mitogens. Deregulation of adhesion dependence results in uncontrolled

cell proliferation and pathologies such as tumor formation. Thus, better understanding of how signaling events initiated by both ECM and GFs regulate cell cycle progression may offer potential strategies for the development of therapeutics.

One signal implicated in GF-mediated regulation of proliferation involves the extracellular signal-regulated kinases 1/2 (ERK1/2) belonging to the mitogen-activated protein (MAP) kinase family (Lewis et al., 1998; Pages et al., 1993). Since these kinases are also activated in response to adhesion to ECM proteins (Chen et al., 1994; Morino et al., 1995), signaling crosstalk at ERK1/2 has been suspected to play an important role in the joint regulation of cell cycle progression by cell adhesion and GFs. In fact, cell adhesion to fibronectin (Fn) has been shown to enhance GF-mediated activation of ERK1/2 (Lin et al., 1997; Miyamoto et al., 1996; Moro et al., 1998; Renshaw et al., 1997), providing a possible mechanism by which adhesion stimulates GF-mediated proliferation. However, this hypothesis remains to be tested quantitatively by examining the relationship between the degree of ERK1/2 signal enhancement and the level of adhesion-dependent cell cycle progression. Does the magnitude of ERK1/2 activation

linearly correspond to level of cell proliferation, and if so, can the effect that adhesion has upon GF-mediated proliferation be predicted directly from an assessment of its effect on ERK1/2 signal intensity? To address these questions, we have examined the effects of adhesion on mitogen stimulation of the ERK2 signal and DNA synthesis in response to systematic variations in level of adhesion and GF stimulation.

In addition to the magnitude of ERK1/2 activation, its dynamics – transient versus sustained – may also govern coordinate regulation of cell cycle progression. In certain fibroblasts, cell adhesion shifts GF-mediated ERK2 activation from a transient to a sustained form, thereby upregulating cyclin D1 (Roovers et al., 1999), which is one step in the eventual hyperphosphorylation of retinoblastoma protein (pRb), its dissociation from E2F transcription factor, and E2F-mediated upregulation of cyclin A expression (DeGregori et al., 1995; Schulze et al., 1996). However, in other cell types, transient ERK1/2 activation correlates with cell proliferation, while sustained activation leads to differentiation (Marshall, 1995). In hepatocytes and PC12 cells, forcing sustained activation of ERK1/2 can inhibit DNA synthesis, while transient ERK1/2 activation promotes DNA synthesis (Tombs et al., 1998). Thus, it remains unclear whether a shift in ERK1/2 signaling dynamics (from transient to sustained) and/or an increase in magnitude of ERK1/2 activation mediate the effects of adhesion on cell cycle progression. Here, we implement a unique approach for quantifying the magnitude of transient signals in order to investigate how the intensity of a short-lived ERK2 signal modulates adhesion-dependent DNA synthesis.

Several previous reports have begun to clarify the effect of adhesion and soluble factors on signaling events downstream from ERK1/2 activation and more proximal to cell cycle regulation, involving cyclins, cdks and cdk inhibitors (Assoian, 1997). Yet, it is not known what quantitative dependence on adhesion and GFs should result from this complex network of signals. Adhesion dependence is often characterized by the failure of soluble mitogens to induce normal cells to proliferate in soft agar. However, when cells are allowed to interact with ECM proteins, the question remains of how the level of adhesion (i.e. receptor occupancy) determines the magnitude of its influence on cell cycle progression. Does the requirement for adhesion resemble a switch that flips between cell cycle progression and arrest, based on the presence versus absence of ECM, respectively? Or, does ECM concentration influence cell cycle progression in a more graded manner with a distinguishable slope, so that the magnitude of cell response may be tuned predictably by adjusting the level of adhesion? Moreover, joint regulation of response by ECM and growth factors has often been described as synergistic. However, the nature of this synergy is undefined: considering the range of signaling crosstalk between ECM and growth factors (Schwartz and Baron, 1999), this synergy may include features extending beyond the simple enhancement of response magnitude (Brugge and McCormick, 1999).

We examine here the quantitative contribution of adhesion to cell cycle progression and the role of transient ERK2 activation in the coregulation of DNA synthesis by the soluble mitogen insulin and the ECM protein Fn. Our major findings are: in the absence of exogenous growth factors, (1) adhesion-mediated activation of ERK2 promotes DNA synthesis in a cell

area-independent manner; (2) a transient form of this ERK2 signal is sufficient to induce this response; (3) the intensity of this signal is a direct, linear predictor of the response level across a broad range of Fn and MEK inhibitor amounts. Under stimulation by both Fn and insulin, (4) costimulation yields not only synergistic enhancement of response magnitude, but also other complex features; (5) these features include increased response sensitivity (from graded to more ‘switch-like’) to changes in insulin concentration and a dramatic ~1000-fold reduction in the dosage of insulin required for half-maximal response among cells adhering to higher Fn density; (6) each stimulus transiently activates ERK2 and these transient signals are required for the overall response; (7) ERK2 is not a point of signaling crosstalk between Fn and insulin, since adhesion to different levels of Fn has no effect on the degree to which insulin activates ERK2; (8) adhesion enhances insulin-mediated DNA synthesis in parallel with augmenting other insulin-induced signals such as insulin receptor substrate-1 (IRS-1) phosphorylation.

MATERIALS AND METHODS

Antibodies and reagents

Human plasma Fn and poly-L-lysine (PL) were obtained from Sigma Chemical Company. The sc-154 anti-ERK2 antibody was purchased from Santa Cruz Biotechnology, and anti- $\alpha 5\beta 1$ integrin adhesion-blocking antibody was obtained from Chemicon. Both anti-IRS-1 antibody from Upstate Biotechnology and anti-phosphotyrosine antibody RC20H from Transduction Laboratories were used in western blotting. Both RGD (GRGDSP) and RGE (GRGESP) peptides were obtained from Life Technologies.

Protein-coating surfaces

Protein coating was always performed on Nunc tissue culture-treated surfaces using the protocol described previously (Asthagiri et al., 1999b). Briefly, Fn or PL diluted in PBS was incubated overnight at 4°C with the surface, either 60 mm dishes or 96-well plates. Surfaces were then washed twice with cold PBS and blocked for 1 hour at 37°C with 2 mg/ml sterile-filtered, heat-inactivated (70°C, 1 hour) BSA in PBS. Prior to use, dishes were washed twice with warm PBS. Since the same protocol and surface was used as described previously (Asthagiri et al., 1999b), the amount of Fn adsorbed onto the surface (density range 0–50×10⁷/mm²) was known for each Fn coating concentration (C_c; in the range 0–10 µg/ml).

Cell maintenance and pretreatment for experiments

Chinese hamster ovary (CHO) cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 4 mM L-glutamine, 1 mM sodium pyruvate and 1% (v/v) 100× non-essential amino acids. Prior to each experiment, cells were serum-starved on 100 mm tissue culture dishes for 18 hours in serum-free medium containing 25 mM Hepes-based DMEM, 4 mM L-glutamine, 1 mM sodium pyruvate, 1% (v/v) 100× non-essential amino acids and 2 mg/ml BSA. Cells were suspended using versene (Gibco) and resuspended in serum-free medium to a concentration of 5×10⁵ cells/ml. They were maintained in suspension for 1 hour to bring adhesion-related signals to a basal level.

Cell stimulation and lysate preparation

For adhesion stimulation, cells were plated on Fn-coated surfaces immediately following their 1-hour suspension. For cases where soluble factor stimulation was also required, serum-free medium was aspirated following 2 hours of cell adhesion to protein-coated substratum and replaced with insulin-containing, serum-free medium.

At desired times following each stimulation, cells were washed once with cold PBS and lysed by adding cold lysis buffer containing 50 mM Tris, pH 7.5, 150 mM sodium chloride, 50 mM β -glycerophosphate, pH 7.3, 10 mM sodium pyrophosphate, 30 mM sodium fluoride, 1% Triton X-100, 1 mM benzamidine, 2 mM EGTA, 100 μ M sodium orthovanadate, 1 mM DTT, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 μ g/ml pepstatin and 1 mM PMSF. The lysis buffer for IRS-1 studies was more stringent, containing 0.5% NP-40 and 0.25% sodium deoxycholate. Cells were scraped into the buffer and allowed to lyse for approximately 15 minutes. Lysates were centrifuged at 14,000 rpm (16,000 g) for 15 minutes, and the supernatant was collected. Micro BCA protein determination (Pierce) was used to determine total protein concentration.

ERK2 activity assay

ERK2 kinase activity was measured using a sensitive in vitro assay performed in a 96-well format as described previously (Asthagiri et al., 1999a). Briefly, sc-154 anti-ERK2 antibody was coated on the surface of Reacti-BindTM protein A-coated wells (Pierce). After washing away unbound antibody from the wells, 25 μ g cell lysate was incubated for 3 hours at 4°C. To measure background, an extra well was incubated with just lysis buffer and was carried through the assay in the same manner as other samples. After washing, each well was resuspended with kinase assay buffer to which was added 40 μ g of myelin basic protein (Sigma). The in vitro reaction was initiated by adding 25 μ M ATP (1 μ Ci [γ -³²P]dATP). After 30 minutes of agitation at 37°C, reactions were quenched with 75 mM phosphoric acid, and quenched reaction contents were filtered through a 96-well phosphocellulose filter plate (Millipore). After washes, ³²P label on each filter paper was quantified, and ³²P measurements were adjusted by subtracting the radioactivity associated with the background sample.

IRS-1 phosphorylation assay

IRS-1 was immunoprecipitated from 100 μ g cell lysate using approx. 4 μ g anti-IRS-1 antibody coated on anti-rabbit IgG beads (Sigma). After four washes, 25 μ l of 1 \times SDS sample buffer was added to the immunoprecipitate, and the sample was boiled for 5 minutes. The entire sample from each immunoprecipitation was resolved by 7.5% SDS-PAGE and transferred onto nitrocellulose membrane. Blots were probed for phosphotyrosine using 1:2500 dilution of RC20H and the SuperSignal[®] Ultra substrate (Pierce). Bands were visualized with the Molecular Imager[®] system (BioRad) and further analyses and quantification were performed with the Multi-Analyst[®] software (BioRad).

Quantification of DNA synthesis

DNA synthesis experiments were performed using Nunc tissue culture-treated 96-well plates for higher throughput. Serum-starved, suspended cells were plated in Fn-coated microtiter wells. In the cases where insulin stimulation was required, insulin-containing serum-free medium was added 2 hours after cell plating, exactly analogous to ERK2 activation and IRS-1 phosphorylation experiments. After 16 hours of adhesion on Fn-coated microtiter wells, 2 μ Ci of [³H]-thymidine (NEN) was added to each well of the 96-well Nunc plate. After 6 hours of incubation at 37°C, medium in each well was transferred to and filtered through a 96-well 0.4 μ M filter plate (Millipore). After washing the Nunc plate once with PBS and transferring the wash fluid to the filter plate, trypsin-EDTA was incubated in each well for 5 minutes in order to suspend the cells. The cell suspension was then transferred and filtered so that the filter plate captured suspended cells while medium containing unincorporated [³H]thymidine was drained. The Nunc plate was again washed with PBS, and the wash fluid containing the remaining suspended cells was filtered through the filter plate.

The filters in each well, now holding suspended cells, were each washed four times with 200 μ l of PBS to remove residual serum-free

medium and incubated for 10 minutes in 70% ethanol in order to lyse cells and precipitate DNA. Afterwards, each well was washed three times with 70% ethanol so that only precipitated DNA material containing incorporated [³H]thymidine was left on the filter. The filter plate was allowed to dry and each filter was punched out into a scintillation vial filled with 0.5 ml of 0.42% NaOCl. After being shaken for 30 minutes, the vials were filled with 3.5 ml of CytoScint fluid. The contents were mixed thoroughly and the amount of [³H]thymidine associated with the filter was quantified using a scintillation counter.

DNA synthesis dose-response curves were fitted to the equation $D = b + [(m - b) \times (S^\eta) / (\epsilon + S^\eta)]$, where D is DNA synthesis level, S is stimulant amount, b is basal DNA synthesis level, m is maximal DNA synthesis level, $\epsilon^{1/\eta}$ is the EC50 stimulant concentration required to induce DNA synthesis at a level half-way between basal and maximal and η is the Hill coefficient.

RESULTS

Transient ERK2 activation governs Fn dose-dependent induction of DNA synthesis

While it is well established that most normal cells depend on adhesion to ECM for entry into S phase even in the presence of soluble mitogens (Assoian, 1997; Schulze et al., 1996), the quantitative nature of this dependence on the amount of ECM ligand is undetermined. To measure this, we quantified DNA synthesis in CHO cells adhered to a substratum coated with graded levels of Fn (Fig. 1A). Initially, to avoid synergistic regulation of DNA synthesis by soluble factors, these experiments were performed under serum-free conditions without exogenous addition of GFs, ensuring that the dominant stimulus for DNA synthesis was adhesion to Fn. Analogous to what has been observed for growth factor stimulation, DNA synthesis is a sigmoidal function of Fn amount. A narrow range of Fn densities from 5-25 \times 10⁷/mm² (C_c =0.1-0.5 μ g/ml) was sufficient to raise the response from a basal to maximal level (Fig. 1A, empty circles). The EC50 Fn concentration required to elicit half-maximal DNA synthesis was approximately 15 \times 10⁷/mm² (C_c =0.25 μ g/ml).

Cell interaction with Fn was necessary to induce DNA synthesis as non-specific cell attachment to a poly-L-lysine (PL)-coated substratum and plating cells onto an Fn-free, BSA-blocked surface failed to initiate this response (Fig. 2). Moreover, the presence of an anti- $\alpha_5\beta_1$ integrin antibody ablated adhesion and DNA synthesis, indicating that $\alpha_5\beta_1$ integrin-Fn interactions specifically mediated this response. Consistent with the fact that Fn engages the $\alpha_5\beta_1$ integrin via its RGD domain (Ruoslahti, 1996), addition of soluble RGD peptide inhibited DNA synthesis whereas soluble RGE peptide was ineffective.

Because signaling through ERK2 has been implicated in regulating growth factor-mediated DNA synthesis, we characterized the role of ERK2 in this adhesion-mediated response. CHO cell adhesion onto an Fn-coated surface transiently activated ERK2 (Fig. 1B, empty circles). To determine whether this short-lived ERK2 activation regulated later DNA synthesis, the MEK inhibitor PD098059 (PD) was used to thwart ERK2 activation (Alessi et al., 1995). Raising the amount of PD drug resulted in stronger constriction of the pulse ERK2 signal (Fig. 1B). Paralleling this inhibition of the ERK2 signal, the PD drug caused a pronounced, dose-

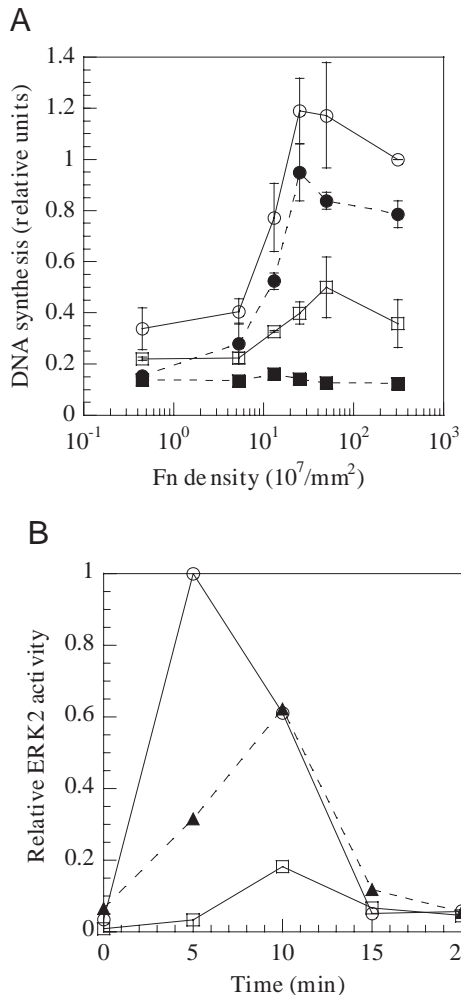


Fig. 1. Transient ERK2 activation regulates Fn-induced DNA synthesis under serum-free conditions. (A) The DNA synthesis response of CHO cells plated in Fn-coated microtiter wells (○) was measured for different Fn-coating levels in the range $0.45\text{--}310 \times 10^7/\text{mm}^2$ ($0.01\text{--}10 \mu\text{g/ml}$). ERK2 inhibition by introducing PD drug (see text) during the 1-hour suspension period prior to plating on Fn-coated substratum yielded a PD dose-dependent reduction in DNA synthesis with $5 \mu\text{M}$ (□) inducing partial inhibition and $50 \mu\text{M}$ (■) PD achieving full inhibition. These effects were specific to PD as the 0.1% DMSO solvent control (●) produced only a minor effect on DNA synthesis. (B) Cells plated on Fn initiated a transient ERK2 response (○) that reached its peak level after 5 minutes and subsided to its basal level within 20 minutes. In addition to delaying the occurrence of the peak ERK2 activity level, treatment with either $0.5 \mu\text{M}$ (▲) or $5 \mu\text{M}$ PD (□) reduced the magnitude of the pulse ERK2 response in a manner parallel to PD-mediated reduction of DNA synthesis.

dependent reduction in Fn-induced DNA synthesis, with complete inhibition attained at the highest PD dose ($50 \mu\text{M}$) (Fig. 1A), revealing that the transient ERK2 signal caused by cell adhesion to Fn regulates DNA synthesis.

DNA synthesis is directly proportional to integrated ERK2 activity independent of cell shape

Since the PD drug offers independent control of ERK2 activity, we employed it to test quantitatively whether the degree of

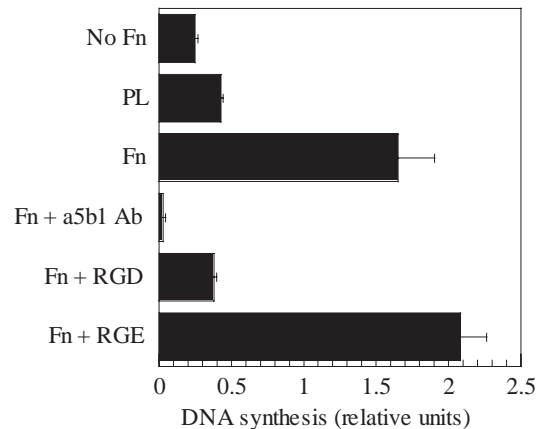


Fig. 2. Interaction between the RGD domain of Fn and the $\alpha 5\beta 1$ integrin is necessary for Fn-mediated DNA synthesis. Cells in serum-free medium were plated on an Fn-coated ($25 \times 10^7/\text{mm}^2$ or $0.5 \mu\text{g/ml}$), PL-coated ($1 \mu\text{g/ml}$), or a non-coated surface. Only attachment mediated by Fn induced DNA synthesis, while failure to attach or non-specific attachment to PL did not elicit this effect. In particular cases, medium was supplemented with anti- $\alpha 5\beta 1$ integrin antibody, 1.25 mM RGD or 1.25 mM RGE. Both RGD and anti- $\alpha 5\beta 1$ integrin antibody blocked the ability of Fn to induce DNA synthesis, while RGE had no effect.

ERK2 activation correlated with the level of DNA synthesis, regardless of Fn amount. A high-throughput assay enabled measurements of the entire time course of ERK2 activity for different Fn coating densities and PD concentrations at which DNA synthesis had already been assessed. As shown in Fig. 1B for a typical Fn coating density, in addition to reducing ERK2 response magnitude, higher doses of PD drug retarded the rate of ERK2 activation and delayed the occurrence of its peak activity level. In the absence of the PD drug, the peak level occurred at approx. 5 minutes, but exposure to $5 \mu\text{M}$ PD extended this to approx. 10 minutes.

In order to relate DNA synthesis to this dynamic ERK2 response, a single-value representation of the entire ERK2 time-course was required. Since the ability of ERK2 to activate its downstream effector would depend not only on the magnitude of ERK2 activity but also the lifetime of that activity, the integral of the ERK2 activation time course was used as a metric to capture quantitatively shifts in both magnitude and kinetics of the signal, as described previously (Asthagiri et al., 1999b). Using this metric, DNA synthesis was found to be proportional to the integrated ERK2 activity (Fig. 3). For any given Fn amount, an increase in PD reduced both the ERK2 signal and DNA synthesis response. Additionally, the data points overlapped along a linear curve fit for different Fn amounts, revealing that fine-tuning the transient ERK2 response determined the level of DNA synthesis irrespective of Fn amount.

In addition to acting through biochemical signals such as ERK2, the ECM has been suggested to regulate cell cycle progression through its effects on cell shape (Chen et al., 1997; Folkman and Moscona, 1978; Price, 1997). We determined whether ERK2 and cell shape exert their effects on DNA synthesis through the same pathway. Because ERK2 activation in our experiments occurred within 20 minutes after plating

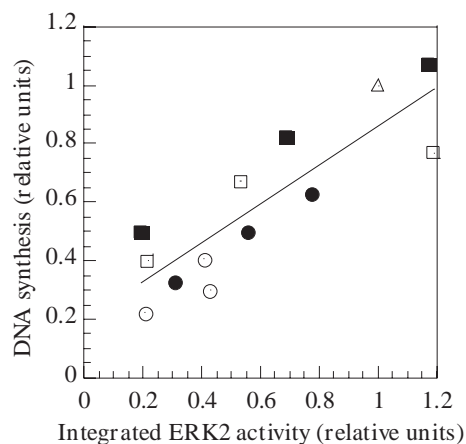


Fig. 3. The level of DNA synthesis is directly proportional to integrated ERK2 activity, irrespective of Fn amount. Both DNA synthesis and the ERK2 time course were measured in response to independently tuning ERK2 signals with PD drug (no pretreatment, 0.5 μM or 5 μM PD) for the following Fn amounts: 5.3 (\circ), 13 (\bullet), 25 (\square), 50 (\blacksquare), 310 (\triangle) $\times 10^7/\text{mm}^2$ (0.1, 0.25, 0.5, 1 and 10 $\mu\text{g}/\text{ml}$, respectively). At each condition, the integral of the ERK2 time course over its 20-minute lifetime was calculated as a metric for this dynamic signal using the trapezoidal rule: Integrated ERK2 response = (5 minutes) \times {Average(E_0, E_5) + Average(E_5, E_{10}) + Average(E_{10}, E_{15}) + Average(E_{15}, E_{20})} where E_i is the ERK2 activity level at time i for $i=0, 5, 10, 15$ and 20 minutes. For a fixed Fn amount (each symbol), an increase in PD concurrently reduced both DNA synthesis and the ERK2 response. Moreover, regardless of Fn amount, DNA synthesis revealed a simple linear dependence on the level of integrated ERK2 activity ($r^2=0.75$).

onto an Fn-coated surface and before any significant cell spreading occurs, it was unlikely that cell shape changes lay upstream of ERK2 activation. This was directly confirmed by the finding that cell area at an early time point (7.5 minutes) after plating on Fn remained at a constant value for different Fn levels (Fig. 4), even though ERK2 activity at this time was strongly dependent on Fn amount (data not shown).

To test the alternate possibility that the level of active ERK2 determined the degree of cell spreading and thereby indirectly affected DNA synthesis (i.e. ERK2 upstream of cell shape), we measured steady-state cell area 6 hours after plating cells on Fn in the presence of different levels of PD drug. In contrast to the effective reduction of the ERK2 signal by the PD drug (Fig. 1B), the drug had no effect on cell area (Fig. 4), indicating that cell shape changes were not downstream of ERK2. Taken together, these data revealed that the Fn-mediated ERK2 signal regulated DNA synthesis in a cell shape-independent manner.

However, since Fn amount affected steady-state cell area, albeit not in a PD-dependent manner, our findings do not preclude the possibility that cell shape regulated DNA synthesis via a parallel pathway (Fig. 4). But, even in this situation, constraining the critical ERK2 signal superceded any pro-DNA synthesis signal from other parallel pathways, including those involving cell shape. Moreover, because of its overriding importance under these conditions, the ERK2 signal is not only essential, but also is a direct, quantitative predictor of the level of DNA synthesis (Fig. 3).

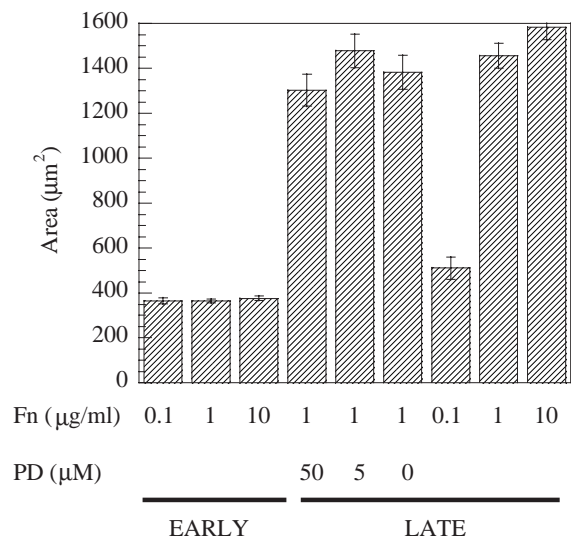


Fig. 4. ERK2 regulates DNA synthesis independently of cell shape. Phase-contrast pictures were taken of cells either 7.5 minutes (EARLY) or 6 hours (LATE) after plating on Fn-coated dishes in the appropriate media: serum-free, serum-free with indicated concentrations of PD (4th and 5th bar), or serum-free with just 0.1% DMSO in the case of 0 PD (6th bar). While cell area at the early time remained at a constant value irrespective of Fn amounts, it varied with the Fn amount at late times, but in a PD-independent manner.

Coregulation of DNA synthesis by Fn and insulin includes dramatic non-reciprocal effects on soluble factor potency

We next extended our analysis to the more physiological scenario in which DNA synthesis-regulating signals would be triggered by both ECM and soluble mitogens such as insulin. Adding a fixed amount of insulin (10 nM) to the medium 2 hours after cells were plated on an Fn-coated surface induced a higher DNA synthesis response than that produced by Fn stimulation alone (Fig. 5, compare open circles and squares). This enhancement was more profound at higher Fn coating densities such as $50 \times 10^7/\text{mm}^2$ ($C_c=1 \mu\text{g}/\text{ml}$), at which the level of DNA synthesis was enhanced approx. 2.9-fold in the presence of both stimuli when compared to Fn alone. Moreover, the degree of this enhancement was grossly underestimated by simply summing DNA synthesis measured in the presence of each stimulus alone (Fig. 5, compare open and closed circles), revealing that the cumulative response to dual stimulation is not simply additive.

In addition to response magnitude, two other properties can help to characterize more completely the dependence of DNA synthesis on Fn and insulin amounts. First, the sensitivity of DNA synthesis to a particular stimulus offers an assessment of how much change in stimulus concentration is required to elevate the magnitude of response from minimal to maximal. At high sensitivity, the response behaves in a switch-like manner, wherein a small increase in stimulus concentration has an acute effect on response magnitude. In quantitative terms, such ultrasensitivity is reflected in a high value for the Hill coefficient (see Materials and Methods; Goldbeter and Koshland, 1981). Another important property is the critical stimulus concentration (EC_{50}) required to elicit half-maximal

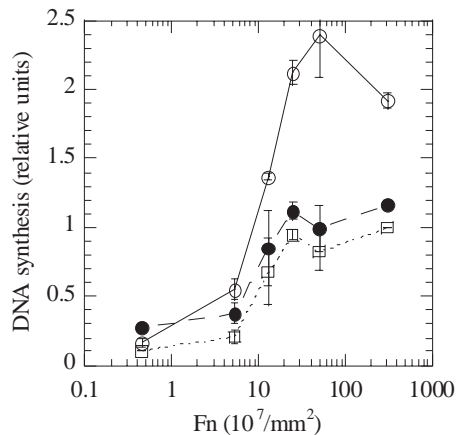


Fig. 5. Synergistic enhancement of insulin-mediated DNA synthesis by Fn. Cells were treated with either serum-free medium or serum-free medium containing 10 nM insulin 2 hours after plating in microtiter wells coated with different levels of Fn ($0\text{--}310\times 10^7/\text{mm}^2$). DNA synthesis for cells plated on Fn and treated with insulin (\circ) was markedly higher than DNA synthesis induced by Fn alone (\square). Moreover, dual stimulation induced a magnitude of response measurably higher than an additive prediction calculated by summing the DNA synthesis response to each stimulus alone (\bullet).

response. A large EC_{50} distinguishes a low potency or weak stimulus that demands a high concentration to induce half-maximal effect.

Coordinated regulation of DNA synthesis by adhesion and a soluble mitogen may incorporate effects not only on response magnitude, but also on response sensitivity to either stimulus and/or stimulus potency. To characterize better this synergistic regulation of DNA synthesis, we implemented a high-throughput microtiter assay, which allowed us to measure DNA synthesis for all combinations and different amounts of insulin and Fn. Except for low insulin amounts ($0\text{--}0.1$ nM), the dependence of DNA synthesis on Fn amount was altered by the presence of insulin (Fig. 6A). At intermediate levels of insulin (1 and 10 nM), DNA synthesis at high Fn levels drastically increased, while still maintaining nearly basal synthesis at low Fn levels. A further increase in insulin (up to 10 μM) was required to appreciably elevate DNA synthesis at low Fn levels. These results show that maximal DNA synthesis was achieved only when both Fn and insulin were present at maximal amounts. This maximal DNA synthesis level attained in the presence of high Fn and insulin concentrations was approx. 3.2-fold less than that observed among cells stimulated with 1% serum (data not shown), indicating that approx. 31% of the cells undergo DNA synthesis in response to maximal stimulation by Fn and insulin.

In contrast to these effects on the magnitude of DNA synthesis, the presence of insulin had little effect on either the sensitivity of the response to Fn or the potency of Fn. Changes in insulin amount did not produce significant variations in the Hill coefficient, which remained between 2.5 to 2.9, and did not drastically affect the surface density of Fn required to elicit half-maximal DNA synthesis or $EC_{50_{Fn}}$, which remained at approx. $10\times 10^7/\text{mm}^2$ (C_c approx. 0.25 $\mu\text{g}/\text{ml}$).

To gain insight into the reciprocal situation wherein Fn may affect insulin-dose response of DNA synthesis, DNA synthesis was replotted as a function of insulin amount for various levels

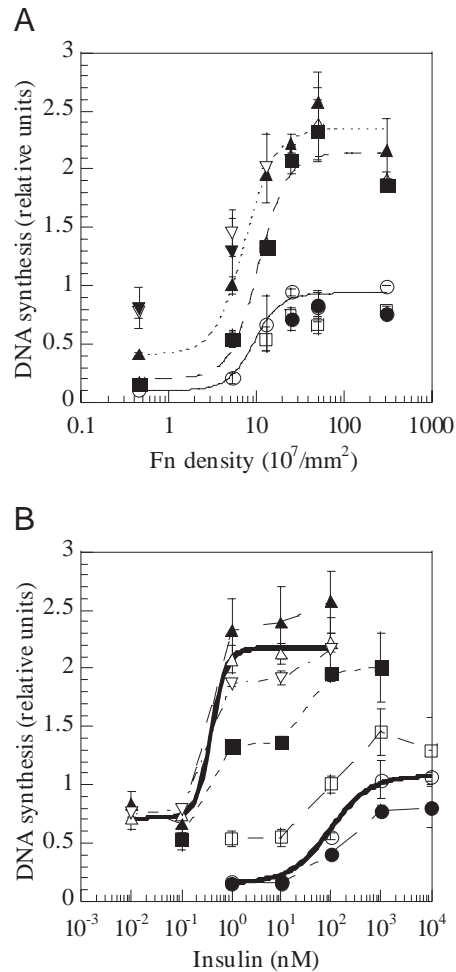


Fig. 6. Complex joint regulation of DNA synthesis by Fn and insulin. (A) Cells plated on different levels of Fn were stimulated 2 hours later with different insulin amounts: 0 (\circ), 0.01 (\bullet), 0.1 (\square), 1 (\blacksquare), 10 (\triangle), 100 (\blacktriangle), 1000 (∇), 10000 (\blacktriangledown) nM. The magnitude of DNA synthesis response is depicted as a function of Fn amount for each insulin concentration. Curve fits to determine $EC_{50_{Fn}}$ and the Hill coefficient were performed for the Fn dose-responses in the presence of 0 (solid line), 1 (broken line) and 100 nM (dotted line) insulin. (B) The same data were replotted to depict the effect that different Fn amounts, i.e. 0 (\circ), 0.45 (\bullet), 5.3 (\square), 13 (\blacksquare), 25 (\triangle), 50 (\blacktriangle), 310 (∇) $\times 10^7/\text{mm}^2$ (0, 0.01, 0.1, 0.25, 0.5, 1, 10 $\mu\text{g}/\text{ml}$, respectively), exert on the dose-response of DNA synthesis to insulin. Here, curve fits to the 0 and $25\times 10^7/\text{mm}^2$ (0 and 0.5 $\mu\text{g}/\text{ml}$, respectively) Fn amounts are depicted by solid bold lines.

of Fn (Fig. 6B). For each level of Fn, DNA synthesis was a monotonic, sigmoidal function of insulin amount. And, similar to insulin's effect on Fn-dose response of DNA synthesis, an increase in Fn amount enhanced the magnitude of insulin-mediated DNA synthesis.

However, in this reciprocal case, effects were not limited to magnitude enhancement. Adhesion to Fn also affected the sensitivity of DNA synthesis to changes in insulin amount. An increase in Fn coating from 0 to $25\times 10^7/\text{mm}^2$ (from $C_c=0$ to $C_c=0.5$ $\mu\text{g}/\text{ml}$) drastically shifted the Hill coefficient from approx. 1 to approx. 2.8 (Fig. 6B; note two heavy lines). This increase in the Hill coefficient reflects the reduced range of insulin amount required to produce a transition from basal to

maximal DNA synthesis at high Fn. While a nearly 100-fold increase in insulin amount was required when Fn was not present, only an approximately tenfold increase in insulin was required to reach maximal DNA synthesis in the presence of $25 \times 10^7/\text{mm}^2$ ($C_c=0.5 \mu\text{g}/\text{ml}$) Fn. This reveals that adhesion to Fn was required for a hypersensitive DNA synthesis response to insulin, suggesting that interactions with the ECM convert GF-induced DNA synthesis from a graded to a switch-like response.

Moreover, an increase in Fn amount reduced the $EC_{50_{\text{Ins}}}$, the insulin concentration required to elicit half-maximal DNA synthesis, as reflected by the leftward shift of the dose-response curves in Fig. 6B as Fn was increased. As shown in Fig. 7, the $EC_{50_{\text{Ins}}}$ strongly depended on the amount of Fn to which cells were adhered. In fact, changes in insulin potency ranged over several orders of magnitude, with low Fn conditions requiring approx. 200 nM insulin to reach half-maximal DNA synthesis while the highest Fn level reduced this requirement to approx. 0.3 nM. Importantly, most of this 1000-fold or so improvement in insulin potency occurs in response to a narrow fivefold increase in Fn density from about 5.3 to $25 \times 10^7/\text{mm}^2$ ($C_c=0.1$ to $C_c=0.5 \mu\text{g}/\text{ml}$), demonstrating the dramatic sensitivity with which adhesion non-reciprocally alters the insulin-dose response of DNA synthesis.

Insulin-mediated transient activation of ERK2 contributes to DNA synthesis

Because ERK2 regulates DNA synthesis in the presence of a single stimulus, Fn, we tested whether ERK2 plays an important role in the more complex regulation of DNA synthesis when two stimuli are present. Similar to stimulation by Fn-mediated adhesion, insulin promoted a transient ERK2 response with a lifetime of approximately 20 minutes, regardless of the amount of Fn to which the cells were adhered (data not shown). Thus, cells stimulated by attachment to Fn and consequent treatment with insulin experienced two ERK2 activation pulses, each occurring immediately after either

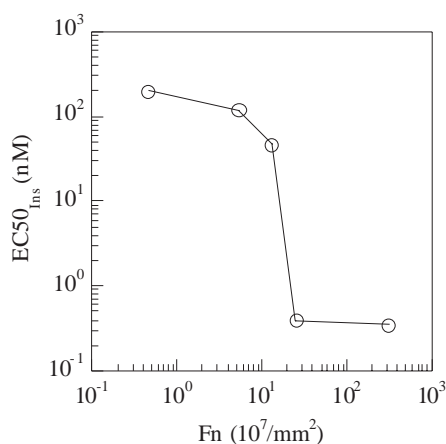


Fig. 7. Cell adhesion to Fn drastically enhances insulin potency for inducing DNA synthesis. For each Fn density, the insulin dose-response of DNA synthesis (Fig. 6B) was fitted to the Hill equation. This yielded the concentration of insulin required for half-maximal DNA synthesis ($EC_{50_{\text{Ins}}}$) at each Fn density. These $EC_{50_{\text{Ins}}}$ values dropped nearly three orders of magnitude over a small range of Fn density, revealing that insulin potency for inducing DNA synthesis is dramatically sensitive to level of Fn to which cells are adhered.

stimulation. Ablation of both ERK2 pulses by PD treatment prior to adhesion to Fn-coated substratum reduced DNA synthesis to a level below that induced by Fn alone (Fig. 8, PD addition 1), suggesting that one or both of these pulses is required for a full effect. To confirm that these two transient ERK2 signals alone were capable of controlling DNA synthesis without later reactivation of ERK2, PD drug was introduced an hour after insulin stimulation to eliminate possible future reactivation of MEK and ERK2. Such PD treatment did not affect the level of DNA synthesis promoted by combined Fn and insulin stimulation (Fig. 8, PD addition 3), confirming that early transient ERK2 signals are fully capable of governing DNA synthesis.

To dissect the portion of the overall response regulated by insulin-mediated ERK2 activation apart from that portion contributed by the Fn-mediated ERK2 signal, PD drug was introduced in the 2-hour time interval between two stimulations, thereby eradicating the insulin-mediated ERK2 pulse while maintaining the Fn-mediated ERK2 signal. The level of DNA synthesis under these conditions matched the level attained by stimulation with Fn alone (Fig. 8, PD addition 2), revealing that failure to induce insulin-mediated ERK2 activation eliminated insulin-mediated enhancement of DNA synthesis.

While this clearly demonstrates that insulin-mediated ERK2 activation is necessary for insulin to contribute to DNA

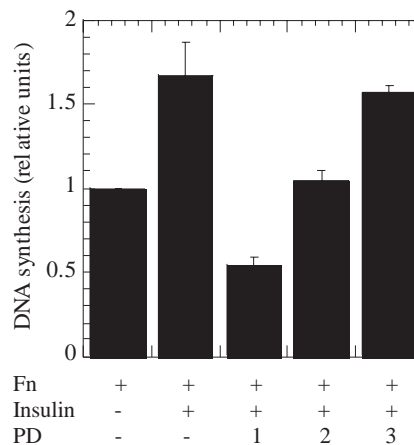


Fig. 8. Relative importance of the Fn- and insulin-mediated pulses of ERK2 activation. In the absence of any PD treatment, stimulation by 10 nM insulin (2nd bar) enhanced DNA synthesis above a level promoted by $310 \times 10^7/\text{mm}^2$ Fn ($10 \mu\text{g}/\text{ml}$) alone (1st bar). Next, $50 \mu\text{M}$ PD was added at different times to selectively ablate ERK2 activation by each stimulus and the consequent level of DNA synthesis response was measured. First, PD was added 1 hour prior to plating on Fn (PD 1) to ablate both Fn- and insulin-mediated ERK2 activation. This effectively reduced DNA synthesis to a level below that supported by Fn alone. Second, PD was added 1 hour after plating on Fn (PD 2), by which time Fn-mediated ERK2 pulse had already occurred, but giving sufficient time for the drug to effectively inhibit later insulin-mediated ERK2 activation. Ablating insulin-mediated ERK2 activation inhibited the insulin-mediated contribution to DNA synthesis. Finally, adding PD drug 1 hour after insulin treatment (PD 3) precluded any later reactivation of ERK2, thereby ensuring that ERK2 undergoes only two transient activations. Yet, these two transient ERK2 signals were fully capable of eliciting DNA synthesis to the full extent induced by insulin and Fn in the absence of any PD drug.

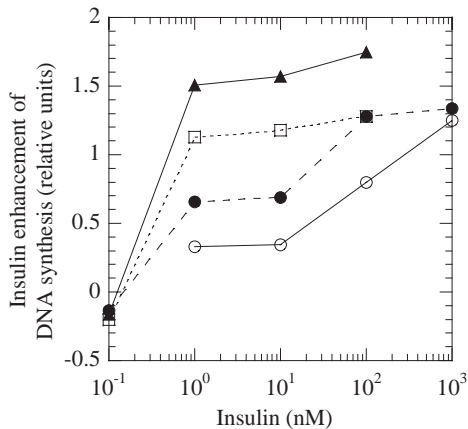


Fig. 9. Insulin-mediated contribution to DNA synthesis is strongly dependent on the level of adhesion. Insulin's contribution to DNA synthesis was calculated by subtracting the level of DNA synthesis promoted by Fn alone (Fig. 1A) from total DNA synthesis supported by dual stimulation (Fig. 6). This insulin-mediated contribution is plotted as a function of insulin concentration at four different Fn densities: 5.3 (○), 13 (●), 25 (□) and 50 (▲) $\times 10^7/\text{mm}^2$ ($C_c=0.1$, 0.25, 0.5, 1 $\mu\text{g}/\text{ml}$).

synthesis, we tested further whether the degree by which insulin enhanced DNA synthesis quantitatively relates to the level of insulin-mediated ERK2 signal. To determine this relationship, we measured DNA synthesis and insulin-mediated ERK2 activation for different doses and combinations of stimuli. Subtracting the magnitude of response to Fn alone from the cumulative DNA synthesis level attained in response to both stimuli offered an assessment of insulin's contribution to DNA synthesis (Fig. 9). For a fixed Fn density, an increase in insulin dose elevates insulin-mediated ERK2 signal (Fig. 10A), corresponding with the concurrent increase in insulin's contribution to DNA synthesis (Fig. 9). However, at each insulin concentration, changes in Fn density altered the magnitude of insulin's contribution to DNA synthesis (Fig. 9), whereas the degree of adhesion had no corresponding effect on the level of insulin-mediated ERK2 signal (Fig. 10A). Thus, adhesion enhanced insulin's ability to induce DNA synthesis without elevating insulin-mediated ERK2 activation, demonstrating that the ERK2 signal, albeit essential, is not a point of crosstalk between Fn and insulin.

Since insulin-mediated ERK2 activation is essential, but yet insensitive to adhesion, it remains unclear whether cumulative DNA synthesis quantitatively relates to level of total ERK2 signal irrespective of stimuli amount, as was the case for sole stimulation by Fn (Fig. 3). To address this question, total ERK2 signal was represented in its most simple form as the sum of signals initiated by each stimulus – that is, the cumulative integral of the two transient ERK2 activation time-courses. As depicted in Fig. 11, the presence of insulin (regimes B and C) alters the simple linear relationship between level of DNA synthesis and integrated ERK2 activity obtained when only Fn is present (regime A). At each Fn density, low level of insulin (0.1 nM) contributes an increase in total ERK2 activity without enhancing DNA synthesis (regime B). Hence, while insulin-mediated ERK2 activation is generally necessary for insulin-mediated enhancement of DNA synthesis, an insulin-mediated ERK2 signal alone is not sufficient to impart higher DNA

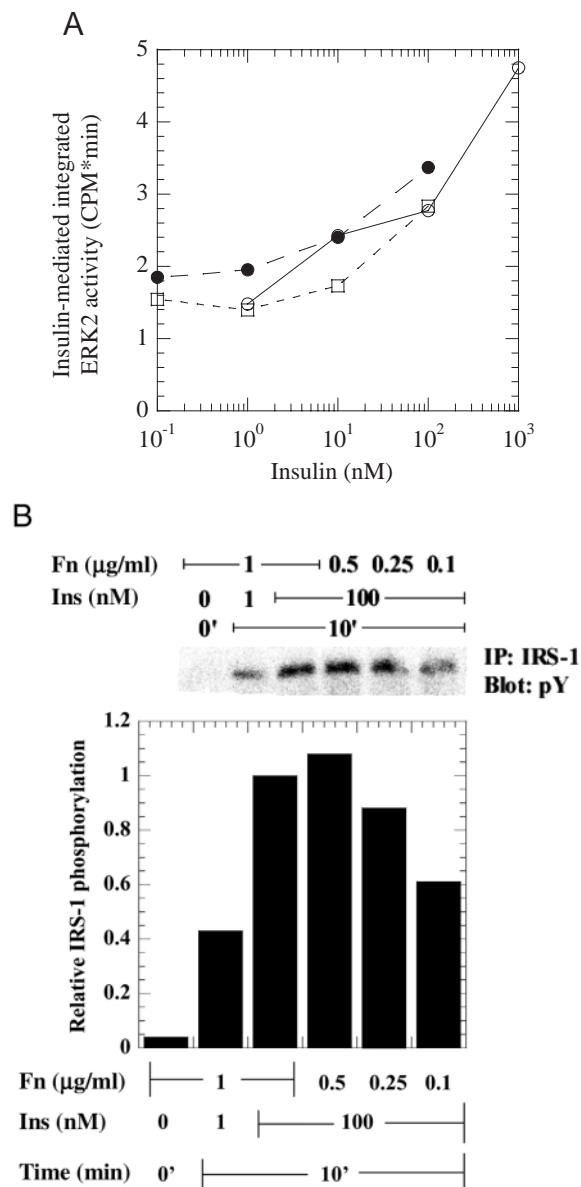


Fig. 10. While insulin-mediated transient activation of ERK2 reveals no dependence on level of adhesion, insulin-mediated IRS-1 phosphorylation was responsive to changes in adhesive surroundings. Cells plated on substratum coated with different levels of Fn were stimulated with insulin 2 hours later. Following insulin stimulation, cells were lysed at the desired times to assess the time course of ERK2 activation and IRS-1 phosphorylation. (A) Insulin doses ranging between 0.1 to 1000 nM were used to stimulate cells seeded on a range of Fn densities: 5.3 (○), 13 (●) and 25 (□) $\times 10^7/\text{mm}^2$ ($C_c=0.1$, 0.25, 0.5 $\mu\text{g}/\text{ml}$). The time course of insulin-mediated ERK2 activation was measured, and its integral was calculated as a metric for the insulin-mediated ERK2 signal. (B) Cells were lysed either 0 or 10 minutes after insulin stimulation and IRS-1 was immunoprecipitated from 100 μg of cell lysate. The phosphorylation level of immunoprecipitated IRS-1 was determined by western blot and quantified using a molecular imager. At a fixed Fn density of $50 \times 10^7/\text{mm}^2$ (1 $\mu\text{g}/\text{ml}$), increasing the insulin dose from 1 to 100 nM elevated IRS-1 phosphorylation. However, unlike ERK2, IRS-1 phosphorylation was sensitive to changes in Fn density (5.3, 13, 25, $50 \times 10^7/\text{mm}^2$; 0.1, 0.25, 0.5, 1 $\mu\text{g}/\text{ml}$) even though the insulin dose was maintained at 100 nM.

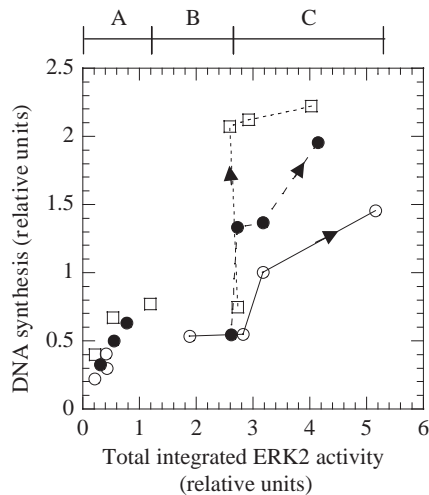


Fig. 11. Dual stimulation with Fn and insulin alters the quantitative dependence of DNA synthesis on integrated ERK2 activity. For stimulation by Fn alone, regime A depicts the relationship between DNA synthesis and total integrated ERK2 activity in response to changes in PD dose and Fn amount: 5.3 (○), 13 (●) and 25 (□) $\times 10^7/\text{mm}^2$ ($C_c=0.1, 0.25, 0.5 \mu\text{g}/\text{ml}$). This regime corresponds to the linear relationship depicted in Fig. 3. With addition of the costimulant insulin (regimes B and C), the total ERK2 signal was calculated by summing the integrals of the transient ERK2 activation time-course initiated by each stimulus. Each data curve represents a particular Fn density: 5.3 (○), 13 (●) and 25 (□) $\times 10^7/\text{mm}^2$ ($C_c=0.1, 0.25, 0.5 \mu\text{g}/\text{ml}$). For each Fn density, the arrowhead on the curve delineates the path of the data as insulin dose is increased over a range 0.1–1000 nM.

synthesis in this regime. Only at higher levels of insulin (1–100 nM) does an insulin-mediated increase in ERK2 signal produce a consequent elevation in DNA synthesis (regime C). However, even in this regime, cells adhered to lower levels of Fn respond more weakly to the same level of total ERK2 activity. This reveals that under dual stimulation the ERK2 signal continues to be essential but is no longer a quantitative predictor of DNA synthesis levels as in the case of stimulation with Fn alone.

IRS-1 phosphorylation serves as a point of signaling crosstalk

To determine whether any other insulin-mediated signal may be more responsive to adhesion levels and therefore serve as a node for signaling crosstalk, we examined the phosphorylation state of IRS-1. While adhesion to Fn itself did not induce IRS-1 phosphorylation, exposure to insulin rapidly initiated this process, which reached a suprabasal pseudosteady state level within 10 minutes (data not shown). As is the case for ERK2 signal, for a fixed Fn coating density of $50 \times 10^7/\text{mm}^2$ (1 $\mu\text{g}/\text{ml}$), stimulation with higher insulin concentration (100 nM versus 1 nM) increased the level of IRS-1 phosphorylation (Fig. 10B). However, unlike the insulin-mediated ERK2 signal, IRS-1 phosphorylation in response to a fixed dose of insulin was sensitive to the level of adhesion. Adhesion to higher Fn density increased level of IRS-1 phosphorylation, even though stimulation involved the same 100 nM dose of insulin. Thus, while insulin-mediated ERK2 signal is unresponsive to the adhesive context, the level of insulin-mediated IRS-1 phosphorylation depends upon the amount of Fn to which cells are adhered. Taken together, these findings demonstrate that

synergy in DNA synthesis response to Fn and insulin stimulation is not a product of signaling crosstalk at the level of ERK2 signaling, but does coincide with coordinated control of IRS-1 phosphorylation.

DISCUSSION

Most normal tissue cells require adhesion to ECM for proper cell cycle progression even in the presence of soluble mitogens. In transformed cells, mutations that override this adhesion dependence allow deregulated traversal through the cell cycle, experimentally characterized by the ability of tumor cells, but not normal cells, to proliferate in soft agar. While this observation asserts the importance of the ECM in regulating proliferation, it does not define the nature of the contribution that adhesion makes to cell cycle progression. To address this issue, we initially quantified the effect of adhesion to Fn on DNA synthesis in the absence of concurrent signals from growth factors.

Although a completely GF-free system is experimentally infeasible due to possible autocrine GF production and/or residual membrane-associated GFs, we find that under serum-free conditions, stimulation by cell adhesion to Fn alone was sufficient to elicit DNA synthesis in CHO cells, with an Fn dose-dependence similar to the sigmoidal dose-dependence of DNA synthesis on GF concentration. Moreover, this response required specific interaction between $\alpha 5\beta 1$ integrin and the RGD domain of Fn, as both anti- $\alpha 5\beta 1$ integrin antibody and soluble RGD peptide blocked this response. The ability of adhesion-based stimulation to induce DNA synthesis suggests that the ECM not only plays a ‘permissive’ role by enhancing growth factor signaling for proliferation, but also by actively initiating signals that can independently control cell cycle progression. Indeed, others have shown that adhesion-mediated signals can trigger expression of immediate-early genes such as *c-fos*, *c-myc*, and *c-jun*, necessary for G_0 – G_1 transition in the absence of soluble factors (Dike and Ingber, 1996). However, full progression through G_1 and into S phase normally requires coordinated signals from soluble factors and adhesion (Assoian, 1997). Therefore, our finding that adhesion alone can stimulate DNA synthesis in our immortalized cell line indicates that transformation may involve not only well-documented deregulation of mitogenic GF signals from prerequisite adhesive signals but also, conversely, deregulation of mitogenic ECM signals from a requirement for GFs.

To identify Fn-induced signals regulating this response, we considered the role of ERK2, partly because it has been implicated in the regulation of DNA synthesis by growth factors. A further reason for studying its role was the finding that ERK2 is activated only transiently in response to cell adhesion to Fn in CHO cells. Despite its short lifetime (approximately 15 minutes), this transient ERK2 signal is essential for adhesion-dependent DNA synthesis measured approx. 15 hours later under serum-free conditions.

Some evidence suggests that ECM-mediated effects on cell proliferation more strongly relate to physical and geometric factors than to biochemical signaling processes (Chen et al., 1997; Folkman and Moscona, 1978; Price, 1997). However, others report that specific integrin-mediated biochemical signals are required for cell proliferation and cannot be compensated by inducing optimal cell shape configurations via

integrins incapable of initiating those critical signals (Davey et al., 1999). In this work, we find that cell shape neither controls the level of ERK2 activation, nor is it affected by pharmacological inhibition of ERK2 signal, the latter of which has also been observed for FG carcinoma cell spreading (Klemke et al., 1997). Thus, in this CHO cell system, a transient ERK2 signal regulates DNA synthesis independent of adhesion-induced effects on cell shape. Moreover, our findings demonstrate that constraining ERK2 supercedes any pro-DNA synthesis signal from parallel pathways involving cell shape.

This critical dependence on the ERK2 signal is observed despite its early and transient activation. While this demonstrates the functional significance of a transient ERK2 signal, the sensitivity of DNA synthesis to changes in strength of signal remains undefined. In order to assess this sensitivity, a proper metric has to be chosen to quantify the strength of a transient signal. Because ERK2 activity always returns to a basal state, its ability to affect DNA synthesis likely involves information transfer onto some downstream component(s) before its complete decay. The simplest model by which this could occur is that at each moment ERK2 is active, it activates a downstream component, which then proceeds to control the ultimate DNA synthesis response. If this downstream component is not subject to the same rapid desensitization as ERK2, then higher ERK2 activity over a longer duration should translate to elevated levels of active downstream component. Because the efficacy of such signal transfer would depend on both the magnitude and duration of ERK2 activity, we propose that the integral of the ERK2 activity time course would be a useful metric or singular quantitative property of this dynamic signal that would permit its correlation to level of DNA synthesis. Indeed, using this metric, DNA synthesis was found to be directly proportional to the integrated ERK2 response across all experimental conditions, demonstrating that the magnitude of DNA synthesis can be 'tuned' simply by adjusting the integrated ERK2 response to the desired level with a pharmacological agent.

Importantly, a unique linear dependence between ERK2 and DNA synthesis exists irrespective of Fn coating density. Thus, the ERK2 signal in cells plated at two different Fn densities can be adjusted to yield the same DNA synthesis response without perturbing other Fn-mediated signals. This should not be misinterpreted to represent the highly unlikely situation that ERK2 is the only signal regulating Fn-mediated DNA synthesis. Rather, our quantitative approach demonstrating that an increase in ERK2 signal corresponds proportionately to elevated levels of DNA synthesis suggests that other necessary promitogenic signals such as pp125^{FAK} (Gilmore and Romer, 1996; Oktay et al., 1999; Zhao et al., 1998) either exist in excess of ERK2 signal or increase in the same manner as ERK2 in response to adhesion to higher Fn amounts so as to not become a limiting factor. In fact, this equal scaling of promitogenic signals with integrin-ligand bond number is further supported by findings from our previous work showing that both pp125^{FAK} phosphorylation and ERK2 activation have the same functional dependence on integrin-Fn bond number under serum-free conditions (Asthagiri et al., 1999b).

Extending beyond stimulation by adhesion alone, we examined the role of ERK2 in regulating DNA synthesis under dual stimulation from both Fn and insulin – a condition that more closely resembles the physiological situation wherein

both the ECM and soluble factors influence cell cycle progression. Introducing insulin 2 hours after plating cells on an Fn-coated surface rapidly activates another transient ERK2 signal and induces a significantly larger DNA synthesis response than Fn alone produced. Both transient ERK2 signals – first stimulated by Fn and again later by insulin – are essential to induce the full level of DNA synthesis. Moreover, inhibiting solely the insulin-mediated ERK2 signal without altering Fn-mediated ERK2 activation allowed DNA synthesis only to the level that Fn alone would support. This demonstrates that costimulation of Fn-adhered cells with insulin enhances DNA synthesis only if insulin contributes its own transient activation of ERK2.

It is important to note that the addition of a soluble factor to adherent CHO cells did not enhance DNA synthesis by now promoting a sustained activation of ERK2. In fact, inhibiting later resurrection of ERK2 activation by introducing PD drug 1 hour after insulin treatment had no effect on the magnitude of response. Thus, the transient nature of these essential ERK2 signals is fully capable of eliciting DNA synthesis in our CHO cell system. However, in some fibroblasts such as NIH3T3 cells, adhesion to matrix was found to enable GF-mediated proliferation by shifting ERK2 activation from a transient to sustained form, which then permitted upregulation of cyclin D1 mRNA, E2F dissociation from pRb, and upregulation of cyclin A (Roovers et al., 1999). Cyclin A expression and cyclin A-cdk2 activity are required for progression through S phase (Girard et al., 1991). Alternatively and consistent with our findings, pRb/E2F-independent mechanisms also control cyclin A expression (Kang and Krauss, 1996), suggesting that the sustained ERK1/2 activation-cyclin D1 pathway may not be the only route to S phase entry. In fact, in adherent hepatocytes and PC12 cells, sustained ERK2 activation opposes DNA synthesis while transient ERK2 signals promote this response by downregulating p21^{Cip1/WAF-1} expression and elevating cdk2 activity (Tombes et al., 1998).

Since joint stimulation by Fn and insulin does not alter the dynamics (always transient) of ERK2 signaling, we investigated whether crosstalk at ERK2 occurred through effects on the magnitude of ERK2 activation. In NIH3T3 cells, integrin-mediated adhesion to Fn enhances EGF-induced ERK2 activation (Lin et al., 1997; Renshaw et al., 1997), providing a possible mechanism by which crosstalk at ERK2 controls adhesion-dependent cell cycle progression. To test directly this possibility, we implemented a high throughput assay to quantify ERK2 time courses at different Fn and insulin levels and applied the above-described integration methodology to calculate the intensity of this transient signal. In our CHO cell system, the level to which insulin activates ERK2 was not dependent on Fn amounts, although insulin's contribution to DNA synthesis was strongly affected by level of adhesion. Thus, adhesion to Fn does not affect either the dynamic character or the intensity of insulin-mediated ERK2 signaling.

Because the lack of crosstalk at ERK2 fails to explain the joint effects of insulin and fibronectin on DNA synthesis, it raises the question of whether other insulin-mediated signals potentially mediate this crosstalk. Indeed, changes in Fn density strongly affected IRS-1 phosphorylation in response to a fixed dose of insulin. In fact, others have delineated several points of crosstalk between ECM- and insulin-mediated signals

including effects on IRS-1 phosphorylation and IRS-1 association with PI3K (Guilherme et al., 1998; Schnell et al., 1997; Vuori and Ruoslahti, 1994). Thus, a subset of these insulin-mediated signals that are also responsive to adhesive surroundings may mediate crosstalk between adhesion and insulin, while insulin-mediated ERK2 activation, albeit essential, does not account quantitatively for response synergy.

This lack of crosstalk at ERK2 activation alters the simple linear dependence of DNA synthesis on integrated ERK2 activity observed in the single Fn stimulation case, producing two quantitatively distinct regimes (B and C, Fig. 11) of response dependence on total ERK2 signal. Further analysis of these new regimes depicts how a signal, which always maintains its essentiality, can perform in quantitatively distinct ways to influence cell response. In regime B, response insensitivity to increased total ERK2 signal results in a threshold effect wherein a certain level of insulin-mediated ERK2 activity has to be mounted before it can effectively impart higher DNA synthesis. Thus, in this regime, insulin-mediated ERK2 activation is essential but not sufficient to enhance DNA synthesis. Only at higher insulin doses in regime C, insulin-mediated ERK2 activity reaches a level that promotes elevated DNA synthesis, but the same degree of signal can yield different levels of response depending on the adhesion context. Therefore, response magnitude cannot be predicted quantitatively by knowledge of total integrated ERK2 activity alone, which is consistent with the finding that signaling crosstalk driving response synergy does not occur at the point of ERK2 activation. Rather, the level of response may be related more directly to other insulin-mediated, promitogenic signal(s) subject to crosstalk by both stimuli such as IRS-1 phosphorylation.

When considering this complexity in the performance of ERK2 alone and the multiple additional signaling pathways likely to impinge upon this response, it is difficult to predict a priori the quantitative synergy with which these two stimuli coregulate DNA synthesis. To ascertain what forms of response synergy result from such a complex network of signals, a high throughput method was implemented to measure DNA synthesis for different combinations and amounts of Fn and insulin. One aspect of the joint regulation involves synergistic enhancement of response magnitude. Significantly, this magnitude enhancement was reciprocal in nature; that is, addition of insulin synergistically enhanced the level of Fn-mediated DNA synthesis, and adhesion to Fn enhanced the level of insulin-mediated DNA synthesis.

However, extending beyond these magnitude effects were strongly non-linear and non-reciprocal effects on response sensitivity and stimulus potency. A quantitative measure of response sensitivity to stimulus is the fold change in stimulus concentration required to shift the response from minimal to maximal. While the presence of insulin did not affect response sensitivity to Fn, adhesion to Fn improved response sensitivity to insulin. Cells plated on surfaces lacking Fn required a nearly 100-fold increase in insulin to increase their DNA synthesis from minimal to maximal. In contrast to this graded response, cells plated on $25 \times 10^7/\text{mm}^2$ ($C_c=0.5 \mu\text{g/ml}$) Fn required less than a tenfold increase in insulin concentration, revealing a more switch-like behavior.

Several mechanisms have been proposed by which a graded stimulus may induce a switch-like response, particularly for the

ERK pathway, which plays a critical role in regulating DNA synthesis in our system (Ferrell, 1996; Goldbeter and Koshland, 1981). However, since these models have considered steady-state signaling responses, it is unclear whether these mechanisms would directly apply to the induction of a switch-like transient signal. Nevertheless, if proposed mechanisms such as the operation of kinases and phosphatases at near saturation (Goldbeter and Koshland, 1981) or the presence of a stoichiometric inhibitor (Ferrell, 1996) were also to produce switch-like transient signals, then the ECM may be involved through its assembly of signaling centers such as focal adhesion complexes. Since these centers localize signaling molecules shared by the ECM and GFs (Miyamoto et al., 1996; Plopper et al., 1995; Schnell et al., 1997; Vuori and Ruoslahti, 1994), they may 'prime' the cell for later GF-induced ultrasensitive signaling responses by concentrating signaling molecules to locally saturating levels or recruiting stoichiometric amounts of inhibitor.

In addition to non-reciprocal effects on response sensitivity, costimulation also has unilateral effects on soluble factor potency. Stimulus potency is inversely related to the concentration of stimulus required to elicit half-maximal DNA synthesis ($EC_{50}^{\text{stimulus}}$). While changes in insulin amount do not affect EC_{50}^{Fn} , adhesion to Fn strongly enhances insulin potency. An increase in Fn amount from 0 to $25 \times 10^7/\text{mm}^2$ ($C_c=0.5 \mu\text{g/ml}$) dramatically reduces EC_{50}^{Ins} from 200 nM to about 0.3 nM. Moreover, most of this 1000-fold shift in insulin potency occurs over a narrow fivefold increase in Fn density, revealing the dramatic sensitivity of this non-reciprocal effect on insulin potency.

These observations on the quantitative effects of costimulation with insulin and Fn have important and widespread implications for our current view of adhesion- and growth factor-mediated coregulation of cell cycle progression. First, in vitro studies assessing the mitogenic responses of growth factor must begin to consider the role of adhesive signaling, specifically the concentration of matrix proteins to which cells are adhered. Small variation in Fn density produces many orders of magnitude change in insulin potency. This suggests efficacy estimations of soluble factors or protein therapeutics under conditions where ECM content is not precisely defined and consistently maintained may disguise in vivo performance of these soluble ligands. Second, in wound healing and tumor development where matrix remodeling is prevalent, our study suggests that even small changes in ECM content through degradation of old components and deposition of new proteins may provide a powerful mechanism for altering cell interpretation of soluble cues.

Finally, our approach shows the value of quantitative measurements in studying signal transduction and cell response. While previous reports have identified that ECM proteins and soluble mitogens synergistically regulate the magnitude of signals and jointly control cell cycle progression (Assoian, 1997; Schwartz and Baron, 1999), our work shows that coregulation of DNA synthesis by these two classes of stimuli has dramatic, non-reciprocal effects on response sensitivity and stimulus potency. Despite this complexity, one mainstay seems to be the role of transient ERK2 activation in governing both Fn- and insulin-mediated DNA synthesis. In addition, a quantitative approach to representing dynamic signals with the integral of their time course was particularly

effective in relating the magnitude of a transient ERK2 signal to a level of downstream cell response, thereby revealing that neither ERK2 dynamics nor its intensity is subject to crosstalk between Fn and insulin. Such relations will play a valuable part in predicting which signaling pathways are potential targets and what degree of manipulation of those targets will be required in order to achieve desired level of cellular response in applications such as disease therapy.

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