Suppression of WEE1 and Stimulation of CDC25A Correlates with Endothelin-dependent Proliferation of Rat Aortic Smooth Muscle Cells

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Proliferation of vascular smooth muscle cells plays a key role in the pathogenesis of several disorders of the vascular wall. Endothelin (ET), a vasoactive peptide that signals through a G protein-coupled receptor, has been linked to mitogenesis in vascular smooth muscle cells, but the mechanistic details underlying this activity remain incompletely understood. In the present study, we demonstrate that ET-dependent mitogenesis in rat neonatal and adult aortic smooth muscle (RASM) cells is accompanied by an increase (up to 10-fold) in CDK2 activity, but not CDK2 protein levels. This effect is blocked almost entirely by PD98059 and U0126, implying involvement of the MEK/ERK signal transduction cascade in the activation. Extracts of ET-treated cells phosphorylate the N terminus of WEE1, an inhibitory kinase, which negatively regulates CDK2 activity through phosphorylation at Tyr15, leading to a decrease in WEE1 activity and a reduction in levels of phospho-Tyr15 in the CDK2 protein. ET also increases expression and activity of CDC25A, the regulatory phosphatase responsible for dephosphorylating Tyr15. All of these effects are reversible following treatment with the MEK inhibitor PD98059. ET also increases levels of CDC2 activity in these cells in association with a decrease in levels of phospho-Tyr15 on the CDC2 molecule. Phosphorylation of WEE1 is linked to ERK while phosphorylation of MYT1 (CDC2-selective inhibitory kinase) is tied to the ribosomal S6 kinase (RSK). In summary, ET controls progression through the cell cycle, in part, by increasing CDK2 and CDC2 activity through the MEK/ERK signal transduction pathway(s). This results from the phosphorylation and subsequent inactivation of two inhibitory kinases (WEE1 and MYT1) that tonically suppress CDK2 and CDC2 activity and activation of a phosphatase (CDC25A) that increases CDK2 activity.

Cell growth and division are important contributors to the pathogenesis of a number of cardiovascular disorders. Hypertension, atherosclerosis, and restenosis post-angioplasty are each characterized by aberrant proliferation of smooth muscle cells in the vascular wall. A detailed understanding of the regulatory controls that govern the growth response in these cells is critical if we are to understand the pathophysiology of these disorders at a level that will permit development of therapeutic strategies directed against specific molecular targets.

CDK2 is the principal cyclin-dependent kinase acting at the G1/S transition. It pairs predominantly with cyclin E at this point in the cycle and is inhibited by several CDK inhibitors including p21/Cip, p27/Kip1, and p57/Kip2. Later in S phase cyclin A replaces cyclin E as the principal CDK2-associated cyclin. Activation of CDK2 is considered a key regulatory event in that it functions as a gatekeeper of the G1/S transition and plays a major role in determining whether a given cell will ultimately progress further in the cycle. In addition to the positive (i.e. cyclin E and A) and negative (i.e. p21, p27, and p57) regulatory mechanisms alluded to above, CDK2 is also heavily regulated through post-translational modification (e.g. phosphorylation). CDK-activating kinase (CAK), a heterotrimer of CDK7, cyclin H, and the regulatory molecule MAT1, phosphorylates CDK2 at Thr160 and promotes activation of the kinase (1). An inhibitory kinase, WEE1, negatively regulates CDK2 activity through phosphorylation at Tyr15 (2, 3). Phosphorylation of WEE1 itself is associated with reductions in its intrinsic kinase activity (2, 4–7). Countering this, a regulatory phosphatase CDC25A dephosphorylates Tyr15 and restores CDK2 activity (8, 9). Thus, the eukaryotic cell has developed several layers of seeming redundant regulatory control to limit access to the G1/S transition and subsequent mitotic activity.

CDC2 (CDK1) takes over as the predominant CDK activity in early G2, continuing through the M phase of the cycle. It pairs predominantly with cyclin B, and to a lesser extent with cyclin A, and it is inhibited by p21/Cip. Like CDK2, CDC2 is heavily regulated through phosphorylation. CDC2 activity is suppressed through phosphorylation of Tyr15 by WEE1 and Thr14 and Tyr15 by a membrane-associated kinase called MYT1 (10). As noted for WEE1, phosphorylation of MYT1 is associated with reduction in its kinase activity (11). MYT1 derives its inhibitory activity both from direct suppression of CDC2 activity and from its ability to sequester CDC2 at cell membranes away from the nuclear compartment (12, 13). CDC25C is the regulatory phosphatase, which is responsible for removing MYT1-dependent phosphates and restoring CDC2 activity (14).

A number of G protein coupled receptors including those for angiotensin, endothelin, and the α-adrenergic agonists possess both potent vasoconstrictor properties as well as pro-mitogenic activity in vascular smooth muscle cells (VSMC) (15). Despite an abundance of information on the signal transduction events...
were cultured in growth media (described above) in 24-well plates. Cells were maintained in medium containing serum substitute for 48 h and then treated with 10⁻⁷ M ET for 20 min. Pretreated cells were exposed to x-ray film.

Expression and Purification of Recombinant Protein from Bacteria—Glutathione S-transferase (GST)-CDK2 (22), GST-c-Myc (T160A mutant) in CDK2 (23), GST-WEE1 (this concentration of WEE1 was induced with 100 μM isopropyl-β-D-galactoside) for 6 h. The cells were harvested, resuspended with 50 mM Tris-Cl (pH 7.5), 2 mM EDTA, 1 mM DTT, and protein inhibitors (Roche Applied Science, 50 mM buffer/tablet) and sonicated. The supernatant was incubated with GSH-Sepharose (Amersham Biosciences) for 2 h at 4°C. The slurry was washed once with lysis buffer and twice with phosphatase reaction buffer. The reaction was initiated by adding 40 μl of cellular lysate was incubated with 1 μg of GST-RB605 (this concentration of RB605 was induced with 100 μM isopropyl-β-D-galactoside) for 20 min. Cellular lysates were used for immunoprecipitation of ERK 1/2 and loaded onto a column. The column was washed with 20 mM Tris-Cl, pH 8.0, 20 mM NaCl, and 5 mM imidazole and then eluted with the same buffer containing 50 mM imidazole.

Immunoprecipitation and Kinase Assay—Cells were treated with the agents indicated for different time intervals. Cells were lysed with lysis buffer (17). 100 μg of supernatant protein was incubated with 1 μg of anti-CDK2, anti-CDK2, anti-cyclin A, anti-cyclin E, anti-CDK4, anti-CDK6, anti-CDK7, anti-WEE1, anti-RSK, or anti-JNK1 antibody and 10 μl of protein G-Sepharose for 1–2 h at 4°C. Immunoprecipitates were carried out as described previously (17) with appropriate substrate (2 μg of histone 1 for measurement of CDK2, CDC2, cyclin A, and cyclin E-associated kinase; 5 μg of GST-RB for measurement of CDC4 and CDK6; 5 μg of GST-CDK2 for measurement of CDK7; 5 μg of myelin basic protein (MBP) or GST-WEE1 for measurement of ERK; 5 μg of GST-c-Myc (T160A mutant) for measurement of RRRSLSRLA (27) and 5 μg of GST-MYT1 for measurement of RSK; and 5 μg of histidine-tagged PA cyclinAGST-mCDK2 complex for measurement of WEE1). To assess phosphorylation of GST-WEE1 through endogenous kinase activity, ET-treated cells were lysed, and lysates were added directly to the kinase buffer. Reaction products were electroblotted on SDS-polyacrylamide gels which were then dried and exposed to x-ray film.

Transfection and Kinase Assay—Adults RASM cells were plated in 10-cm dishes and grown to 70% confluence. Transfection was carried out with Lipofectin reagent (Invitrogen) using a protocol recommended by the manufacturer. 9 μg of wild type ERK or dominant-negative MEK expression vector (25) or control vector was introduced into each culture. The DNA-liposome suspension was incubated with the cultures for 5–6 h at 37°C in Opti-MEM I reduced serum medium (Invitrogen). The suspension was then removed and replaced with serum substitute medium for the ensuing 48 h, at which point cells were treated with ET for 20 min. Cellular lysates were used for immunoprecipitation of ERK and WEE1. Their respective kinase activities were measured as described above.

Cdc25a Phosphatase Assay—Adult RASM Cells were exposed to PD098059 or vehicle for 1 h and then treated with ET for 24 h. 100 μg of cellular lysate was incubated with 1 μg of anti-Cdc25a antibody and 10 μl of protein G-Sepharose for 1–2 h at 4°C. Immunoprecipitates were washed once with lysis buffer and twice with phosphate buffer (30 mM Tris-HCl, pH 7.0, 75 mM NaCl, 1 mM EDTA, 0.33% bovine serum albumin, and 1 mM DTT) (28) at 4°C. The reaction was initiated by adding 40 μl of 0.5 M fluorescein monophosphate (OMP) in 150 μl of reaction buffer and incubated at room temperature for 1 h. The phosphatase catalyzes the metabolism of OMP to the fluorescent product, 2′-O-methylfluorescein (OMF). Fluorescent emissions from the hydrolysis of OMF to OMF were measured at room temperature using a Photon Technology Inc. Quantum Master Fluorometer (29) with an excitation filter of 485 nm and emissions filter of 530 nm. Data were fit to the appropriate equation using nonlinear least-squares analysis in ProFit 5.1.0 (Quantum Soft).

Exptl and Stat Methods—Cell lysates (20 μg) were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk and probed with anti-p-p38, anti-cyclin E, anti-cyclin A, anti-cyclin D1, anti-CDK4, anti-CDK6, anti-CDK2, anti-Cdc25a, anti-p57, anti-p27, anti-p21, anti-p16, or...
anti-WEE1. A horseradish peroxidase-conjugated second antibody was employed to detect immunoreactive bands using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Biosciences). In selected cases immunoprecipitation was carried out prior to Western blot analysis. Cellular lysates (200 μg) were incubated with anti-CDK2 or anti-CDC2 antibody and protein G-Sepharose for 1–2 h at 4°C. Immunoprecipitates were collected and then boiled for 5 min. Denatured CDC2 and CDK2 protein were subjected to SDS-PAGE. Western blot was carried out as described above with anti-CDC2-phospho Tyr15 (Cell Signaling Technology, Beverly, MA). This antibody reacts with phospho-Tyr15 in both CDK2 and CDC2 (30).

Statistical Analysis—Data were evaluated using one-way analysis of variance and the Newman-Keuls test to assess significance.

RESULTS

ET has been reported to stimulate cell proliferation in a number of in vitro models, including vascular smooth muscle cells (15), albeit not under all conditions (16, 31, 32). As shown in Fig. 1A, [3H]thymidine incorporation in our neonatal RASM cells increased 5-fold in response to treatment with ET (10−7 M) for 24 h. Note that basal incorporation of [3H]thymidine was readily detectable in the untreated cultures (see Fig. 1 legend).

The increment in DNA synthesis was accompanied by true mitogenic activity with roughly a 2-fold increment in cell number seen following 24 h of treatment with the peptide (Fig. 1B).

ET has been shown to activate a number of different pathways in vascular smooth muscle cells (33, 34). Among these the extracellular signal-regulated kinase (ERK) has been most closely associated with the pro-mitogenic activity of the peptide (17–19). As shown in Fig. 2A, ET elicited approximately a 6-fold increment in ERK activity in the RASM cells which was readily suppressed by co-incubation with the MEK (ERK kinase) inhibitors PD98059 and U0126. The stimulation of ERK was accompanied by a 6-fold increment in p90 ribosomal S6 kinase (RSK) activity, a downstream target of ERK (Fig. 2B) and, in similar fashion, the increase in RSK activity was abolished following co-incubation with the MEK inhibitors (Fig. 2C). A 4-fold ET-dependent increment in [3H]thymidine incorporation was similarly reduced, albeit incompletely (~60–70% inhibition), with the MEK inhibitors (Fig. 2D). This was accompanied by a similar partial reversal in the ET-dependent increment in cell number (Fig. 2E). ET also effected a stimulation of CDK2 activity was measured in an in vitro kinase assay using histone 1 as substrate. Representative autoradiographs and/or pooled data are shown (n = 3). **, p < 0.01, *, p < 0.05 versus control.

Fig. 2. MEK inhibitors, PD98059 and UO126, decrease ET-stimulated ERK activity, RSK activity, [3H]thymidine incorporation, cell number, and CDK2 activity in RASM cells. Panel A, neonatal RASM cells were pretreated with 10−7 M PD98059 or 10−6 M UO126 (all subsequent experiments use same concentrations of these two compounds) for 1 h, then treated with ET for 10 min. Immunoprecipitation of ERK was performed and immune complex kinase assays were carried out using MBP as substrate. Panel B, cells were incubated with ET for different time intervals. Immunoprecipitation of p90 RSK was carried out and immune complex kinase assays were performed using RRRLLSSLRA peptide as substrate. Panel C, cells were pre-incubated with PD98059 or UO126 for 1 h, then treated with ET for 5 min. Immunoprecipitation was performed and RSK was measured as described above. Panels D and E, cells were pretreated with PD98059 or UO126 for 1 h and then treated with ET for 24 h. [3H]thymidine incorporation (panel D) and cell number (panel E) were measured. Panel F, cells were incubated with PD98059 or UO126 for 1 h before the addition of ET for 16 h. Cellular lysates were immunoprecipitated with anti-CDK2, anti-cyclin E, or anti-cyclin A antibodies. CDK2 activity was measured in an in vitro kinase assay using histone 1 as substrate. Representative autoradiographs and/or pooled data are shown (n = 3). **, p < 0.01, *, p < 0.05 versus control.
activity, measured either directly in immune complex kinase assays or indirectly as cyclin E- or cyclin A-associated kinase activity (Fig. 2F). This increase was nearly completely abrogated by the MEK inhibitors implying a mechanistic link between ET stimulation of ERK and the subsequent activation of CDK2 and accompanying mitogenic activity. Neither CDK4, CDK6 (each of which interacts with cyclins D1–4 at an earlier point in the cell cycle) nor CDK7 (the CDK-activating kinase or CAK) activity were affected by ET treatment (data not shown).

The neonatal RASM used here have the advantage that they can be passaged in culture for an extended period of time; however, it is formally possible that their growth characteristics may not adequately mirror those found in adult cells. To exclude the possibility that the observed effects represent an artifact intrinsic to the neonatal system, we carried out parallel studies using RASM cells cultured from adult rat aorta. As shown in Fig. 3A, ET treatment led to a similar, albeit less robust (3.5 versus 5-fold) increment in $[^{3}H]$thymidine incorporation which was blocked (~50%) by the MEK inhibitor PD98059 (Fig. 3A). ET treatment also led to a time-dependent increment in ERK and CDK2 activity (Fig. 3, B and C) and, in both cases, these inductions were blocked by PD98059 (Fig. 3, D and E). These findings indicate that the ET-dependent activation of CDK2 operates through a MEK-dependent signaling event in both neonatal and adult RASM cells. The qualitative features of individual inductions are similar in the two cell types.

To explore more closely the mechanism responsible for the increase in CDK2 activity, we measured levels of several key cell cycle regulators using conventional Western blot analysis. Levels of CDK2, cyclin E and cyclin A, the $G_{1}$ cyclins typically associated with increased CDK2 activity, were readily detectable in the neonatal RASM cultures (these cells divide at a modest rate under basal conditions, see Fig. 1) but were unaffected by ET treatment (data not shown). Levels of cyclin D1 and its cognate cyclin dependent kinases, CDK4 and CDK6, were also unchanged following ET treatment, as were the CDK inhibitory proteins p57, p27, and p16INK while p21 was modestly increased (data not shown).

In the absence of increases in CDK2-associated cyclins (cyclins A and E), increases in levels of CDK2 itself, increases in activity or levels of the CDK activating kinase (CAK or CDK7) and no evidence for reductions in levels of the CDK2 inhibitors p21, p27, or p57 (p21 levels, in fact, increased as noted above), we were left to explore other mechanisms to account for the
ET-dependent stimulation of CDK2 activity. Activity of CDK2 and that of its G2 counterpart CDC2 are regulated by two opposing kinase activities. CAK/CDK7 phosphorylates Thr160 in CDK2 (Thr161 in CDC2) promoting activation of the kinase while phosphorylation of Tyr15 in CDK2 by a unique kinase termed WEE1 leads to suppression of CDK2 activity (2–7). For CDC2 this latter inhibitory function is subserved by a combination of WEE1, which phosphorylates CDC2 at Tyr15 and a second kinase called MYT1, which phosphorylates CDC2 at Thr14 and Tyr15 and blocks its activity (11, 35). Control of WEE1 or MYT1 activity occurs through phosphorylation (N-terminal domain of Wee1 and C-terminal domain of Myt1) with subsequent reduction in their respective inhibitory kinase activities (2–4, 7, 11).

We examined the ability of ET to promote phosphorylation of WEE1 in the neonatal RASM cell cultures. As shown in Fig. 4A, extracts from ET-treated RASM cells effected a 4–5-fold increment in GST-WEE1 phosphorylation compared with control cells. This occurred within 20 min following administration of the peptide and slowly decayed back toward baseline over the next 3 h. Endogenous WEE1 protein levels in the extracts, assessed using conventional Western blot analysis, were unchanged over the same time interval. Importantly, the ET-dependent increment in WEE1 phosphorylation was completely reversed by the MEK inhibitor PD98059 (Fig. 4B) suggesting that the observed increment in CDK2 activity is mediated by a MEK/ERK-dependent phosphorylation and inactivation of WEE1. This latter point was explored more fully through direct measurement of WEE1 activity in extracts of ET-treated versus untreated RASM cells.

As shown in Fig. 5A, IPs of WEE1 collected from extracts of untreated cells phosphorylated a modified CDK2 substrate (mutated at Thr160). ET decreased the level of WEE1-dependent CDK2 phosphorylation by 70%. This effect also was completely reversed by the MEK inhibitor PD98059. Furthermore, treatment with ET led to a reduction in the level of phosphorylation of CDK2 at Tyr15 (the target of WEE1) (Fig. 5B) and, once again, this was reversed by PD98059. Extrapolating these analyses to adult RASM cells demonstrated a similar ET-dependent phosphorylation of WEE1 (Fig. 6A) and coincident reduction in WEE1 activity (Fig. 6B). In each instance the ET effect was reversed by co-incubation with the MEK inhibitor (Fig. 6, A and B). Additional support for this model was obtained through transfection of wild-type ERK or dominant negative MEK expression vectors into adult RASM cells. Overexpression of ERK led to the expected
increase in ERK activity, assessed using GST-WEE1 as a substrate (Fig. 6C). This was only modestly increased by treatment with ET. Transfection of dominant negative MEK [MEK (−)] had little effect on basal activity but reduced the ET-dependent increment in ERK activity by more than 50%. Of note the ERK transfectants demonstrated a significant reduction in WEE1 activity, using GST-mCDK2 as a substrate, which was equivalent to but not additive with that effected by ET (Fig. 6D). Cotransfection of dominant negative MEK led to partial reversal of the ET-dependent inhibition. Collectively, these data make a compelling argument that ET stimulates CDK2 activity and promotes cell cycle progression in RASM cells at different developmental stages, at least in part, through an ERK/MEK-dependent suppression of the inhibitory kinase Wee1.

If inhibition of WEE1 was entirely responsible for the increment in CDK2 activity and subsequent increment in proliferative activity, we would predict that the kinetics of the WEE1 inhibition should closely parallel those of the CDK2 induction. In fact, the nadir of the WEE1 inhibition occurs at 30–60 min. while CDK2 activation does not approach maximal levels until 12–16 h following ET treatment. We reasoned that the phosphorylation status of Thr14 and Tyr15, respectively, on the CDK2 molecule might be controlled by factors independent of WEE1. We focused attention on CDC25A, a protein phosphatase that is known to be responsible for removing phosphate groups from Thr14 and Tyr15, inferentially activating the CDK2 protein (8, 9). As shown, in Fig. 7A, Western blot analysis demonstrated a time- and ET-dependent stimulation of CDC25A levels in adult RASM cells. This stimulation reached statistical significance after 8 h and was still operative at 24 h following ET addition. Again, inclusion of PD98059 blocked the ET-dependent increment in CDC25A levels in these cells (Fig. 7B). Independent analysis confirmed that ET also increased the phosphatase activity of CDC25A in the RASM cells and, once again, this increase was neutralized by inclusion of the MEK inhibitor (Fig. 7C).

Finally, we examined the effect of ET on CDC2, the dominant cyclin dependent kinase in the G2/M phase of the cell cycle. As shown in Fig. 8A, ET treatment led to a 6-fold increment in CDC2 activity which was nearly completely suppressed by treatment with the MEK inhibitors. Levels of CDC2 protein were unaffected by ET treatment (data not shown). The stimulation of CDC2 activity was accompanied by reduction in levels of phospho-Tyr15 CDC2 (Fig. 8B) likely reflecting the reduction in WEE1 and possibly MYT1 activity. Again, PD98059 reversed the ET-dependent reduction in phospho-Tyr15 CDC2. The lack of available antibody capable of reacting with rat MYT1 precluded direct assessments of MYT1 levels or
activity; however, we were able to assess the capacity of the activated ERK/RSK signal transduction pathway to promote phosphorylation of MYT1 and WEE1 in vitro. As shown in Fig. 9A, IPs of ERK from extracts of ET-treated RASM cells phosphorylated GST-WEE1 with the net increment falling in the range of 3–4-fold. IPs of RSK from the same cells (RSK is activated by ET in these cultures in MEK-dependent fashion; see Fig. 2) were devoid of activity as were IPs of c-JUN N-terminal kinase (JNK) and p38 mitogen activated protein kinase (MAPK), both of which are also activated by ET treatment in this system (data not shown). Noteworthy, ERK IPs from the same ET-treated cells were capable of only weakly phosphorylating GST-MYT1 while IPs of RSK effected a robust phosphorylation of the protein (Fig. 9B). Once again, IPs of JNK and p38 MAPK from control or ET-treated cells were devoid of activity. Collectively, these data suggest that while ET uses the integrated MEK/ERK/RSK pathway to regulate proliferation in cultured RASM cells; the relevant effectors differ to some degree depending on which portion of the cell cycle is targeted for activation.

**DISCUSSION**

G protein-coupled receptors play an important role in the regulation of VSMC physiology. Vasoactive peptides that operate through these receptors, like angiotensin II and endothelin, control vasomotor tone through their vasoconstrictor properties and are thought to stimulate mitogenesis of smooth muscle cells within the injured or diseased vascular wall (15). This mitogenic activity contributes to the vascular remodeling that accompanies disorders like atherosclerosis, hypertension, and restenosis post-angioplasty. Despite the obvious clinical importance of this process, our understanding of the detailed molecular events linking these ligands and their receptors to enhanced proliferative activity remains incomplete.

It is important to note that AII and ET are not uniformly promitogenic in all settings (16, 31, 32). Jahan et al. (16) have suggested that ET and AII function as progression factors at the G1-S transition, rather than as competence factors, like platelet-derived growth factor (PDGF), which are capable of...
transitioning cells from G₀ into G₁. In support of this model, Yang et al. (31) demonstrated that under conditions where ET itself lacks promitogenic activity, it remains capable of amplifying the response to PDGF-BB. The fact that ET proved capable of promoting mitogenic activity in our studies implies that at least a subpopulation of cells in our cultures were actively cycling, thereby providing a substrate for progression. This hypothesis is supported by the level of basal [³H]thymidine incorporation in the untreated cultures (Fig. 1) and readily detectable cyclin D₁ levels and CDK4 and CDK6 activity that did not change with ET treatment. This activity is likely engendered by additives (e.g. insulin) present in our culture medium or by autocrine/paracrine factors produced locally by the RASM cells. Jahan et al. (16) noted the presence of similar cell cycle progression in subcultured VSMC without the addition of exogenous growth factors, leading them to favor the autocrine/paracrine model.

The present study demonstrates that CDK2, a key regulator of the G₁/S transition, is controlled by ET-dependent ERK activation in the RASM cell and provides an explanation for its ability to promote cycle progression. This induction is selective and does not result from a generic increase in cell cycle activity since levels and activities of a number of other cell cycle effectors were unaffected by ET. The increase in CDK2 activity arises not from increased CDK2, cyclin A/E expression or CDK7 activation nor from suppression of the CDK2 inhibitors (p21, p27, and p57) but from ERK-dependent phosphorylation and subsequent inhibition of the WEE1 kinase and an increase in CDC25A protein levels and phosphatase activity, resulting in a reduction of Tyr¹⁵ phosphorylation of CDK2. The non-overlapping kinetics of the WEE1 inhibition and the CDC25A induction implies that the former is of primary importance during the initiation phase of the CDK2 induction while the latter plays a more important role in the consolidation phase, rising in parallel with CDK2 activity.

Phosphorylation of WEE1 by a variety of kinase activities has been associated with suppression of WEE1 activity (2, 4–7). This includes phosphorylation of the C-terminal domain by a protein called NIM1 (36) and mitosis-specific phosphorylation of the N-terminal domain by an undefined kinase activity (6). Walter et al. showed previously that a mitogen-activated protein kinase was capable of phosphorylating the N terminus of WEE1 in Xenopus egg extracts; however, this was linked to a slight increase rather than the reduction in WEE1 kinase activity (37) reported here. A link between ERK and CDK2 activation has been reported previously; however, the mechanism underlying this association was not described (38, 39). Interestingly, Yang et al. (31) reported that ET did not stimulate CDK2 activity under culture conditions where it failed to increase [³H]thymidine incorporation, implying that the presence of competence factors may also be permissive for ET-dependent stimulation of CDK2 activity. To our knowledge the current study represents the first report that a G protein-coupled receptor ligand (i.e. ET) controls mitogenic activity through an ERK-mediated phosphorylation and inactivation of
WEE1 and/or activation of CDC25A.

Although we have not explored potential regulators of CDC2 in detail, it appears likely that its activity, like that of CDK2, is controlled through ERK-dependent inactivation of inhibitory kinase activity. One component of this inactivation presumably involves the same ERK-mediated suppression of WEE1 activity since the latter has been shown to phosphorylate and suppress the activity of CDC2. A second component appears to derive from ERK-dependent activation of RSK, which phosphorylates the CDC2-selective inhibitory kinase MYT1 (Fig. 9B and Ref. 11). The low level phosphorylation of MYT1 by the ERK IPs (Fig. 9B) identified in our study may reflect residual RSK, which is often found complexed with ERK in the cell (40). The association of CDC2 with the membrane-bound MYT1 also provides an element of temporal flexibility if the kinetics of ERK versus RSK induction differ considerably within the context of the intact cell. RSK may also provide a venue for communication with other signal transduction pathways that are ERK-independent (41).

In summary, ET operating through the MEK/ERK/RSK signal transduction pathway controls cell cycle progression in RASM cells through activation of the CDKs controlling two critical “transition points.” This is accomplished through phosphorylation and subsequent inhibition of two inhibitory kinases (WEE1 and MYT1) that tonically suppress CDK2 and CDC2 activity and activation of a stimulatory phosphatase (CDC25A) that increases CDK2 activity (Fig. 10). Such information may prove useful in designing therapeutic strategies to target smooth muscle cell mitogenesis in disorders characterized by hyperproliferative states in the vascular wall.

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