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p42-MAP kinase is activated in EGF-stimulated interphase but not in metaphase-arrested HeLa cells

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Abstract It is known that cellular signals produced in response to an inappropriate spindle formation cause the cell to be arrested at metaphase (M) in the cell cycle. We report here that the 42-kDa isoform of MAPK (ERK2) was tyrosyl-phosphorylated and activated in response to epidermal growth factor (EGF) in interphase but not in M-arrested HeLa cells. However, the basal level of activity of M-arrested cells was higher than that of interphase, although the overall tyrosyl phosphorylation content was small. Further, the EGF receptor and its associated proteins GTPase-activating protein and phospholipase C were phosphorylated in M-arrested cells to a lower extent than they were in interphase. This implies that in spite of its high level of basal activity, the scarcity of MAPK activation in mitosis in response to EGF stems from an early impairment of phosphorylation of the receptor and neighboring proteins. The biological significance of these results underlies the importance of keeping the cell sheltered from extracellular signals when it undergoes division.

Key words: Signal transduction; Epidermal growth factor; Mitogen-activated protein kinase; Mitosis; HeLa cell

1. Introduction

Binding of epidermal growth factor (EGF) to its receptor induces a rapid receptor dimerization resulting in cross-phosphorylation of the subunits on tyrosine residues and a concomitant activation of the tyrosine kinase enzymatic activity within the intracellular domain. The multi-phosphorylated region provides specific binding sites for cytosolic enzymes containing SH-2 and SH-3 domains (e.g. phospholipase C (PLCγ1), GTPase-activating protein (GAP)) that form a complex with the receptor and become tyrosyl-phosphorylated and activated. Activation of Ras, and Raf and MEK kinases, eventually activates mitogen-activated protein kinases (MAPKs). The $p42^{mapk}$ isoform is a key player in the signaling mechanisms that transduce mitogenic signals from an activated receptor kinase to cell interior molecules including the ribosomal S6 kinase (R6K) [1,2].

MAPK plays a role in: (a) the G_0/G_1 transition that is initiated by EGF and related growth factors in proliferating cells [3–5]; (b) the reentry into the cell cycle of meiosis-arrested *Xenopus* oocytes as induced by extracellular signals [6]; and (c) response to inappropriate spindle assembly during mitosis of either proliferating cultured cells [7] or cycling *Xenopus* eggs [8,9]. In fact, activation of MAPK leads to both a metaphase arrest in oocytes, embryonic or adult cells [10,11] and a G_2 arrest in *Xenopus* eggs [12]. MAPK is activated by microinjection of maturation-promoting factor (MPF) to cell-free extracts of interphase eggs [13]. It has also been demonstrated that in addition to the spindle assembly checkpoint, activation of MAPK at inappropriate times prevents normal entry into M phase [14].

In general, these studies have relied upon the addition of constitutively active Ras, Mos or MAPK/MEK mutants as activators of the MAPK signaling pathway. In spite of this and of the presence of an observable spindle arrest, it has been difficult to observe an activated MAPK during the M phase of somatic cell cycles. Additionally, it is not well known whether an active MAPK, if present during the M phase, will respond to extracellular signals like those inducing G_0 exit or meiosis resumption. Several observations support the concept that mitosis is a period in which cells are insulated from environmental regulation: cell adhesion, membrane transport, arachidonic acid and Ca^{2+} influx are abolished in several cell lines [15,16]. We report here that MAPK was selectively blocked to respond to an extracellular stimulus during metaphase, and that this was due, at least partially, to a lower phosphorylation of the receptor. Since MAPK plays such a central role in signal transduction, it can be beneficial for the cell to turn off certain functions that might need to remain inactive during the critical stages of cell division.

2. Materials and methods

2.1. Materials and antibodies

Recombinant human EGF was purchased from Chiron (Emerville, CA); IgG agarose beads, colcemid (demecolcine) and MBC were from Sigma (St. Louis, MO); DFP was from Aldrich (Milwaukee, WI); Immobilon PVDF membranes were from Millipore Corporation (Bedford, MA); electrophoresis chemicals were from Bio-Rad Laboratories (Richmond, CA); [γ-^{32}P]ATP (30 Ci/mol) was from Amersham Life Sciences (Arlington Heights, IL); anti-$p42^{mapk}$, anti-PLCγ1 and anti-GAP antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY); anti-PY and anti-EGF•R were from Chemicon International Inc. (Temecula, CA).

2.2. Cell culture and synchronization

The model used in this study comprised exponentially growing, cultured human cervical carcinoma HeLa cells: 'interphase', and colcemid-treated cells that were taken off the cell culture dish 'mitosis', as follows. HeLa-S3 cells were seeded at 1×10^5 cells/ml on 100 mm Falcon tissue culture dishes and grown in monolayers in F-12 medium supplemented with L-glutamine and 7% FBS as described [16]. Mitotic cells were obtained by continuous exposure of monolayers to 0.1 μg/ml colcemid during 15 h. The loosely attached, rounded-up mitotic cells were collected the following day by gentle shaking of the plates and concentrated by centrifugation. Fluorescent microscopy of Hoechst 33258-stained cells showed a ≥95% mitotic index. In control experiments, removal of colcemid makes cells capable of again attaching to the cell culture dishes and ∼80% progress into G_1. To create interphase populations similar to those of M-arrested, normally growing cells were transferred from the Falcon tissue culture plastic to Petri dishes on which growth occurs in suspension. Cells remain

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rounded after 24 h culture and are easily collected without the need of trypsin treatment. The interphase population contained 5–10% mitotics at any given time.

2.3. Preparation of cell extracts and immunoprecipitation

This was done as indicated previously in [17] with slight modifications. Briefly, at the time of the experiment, cells were incubated at 37°C and stimulated with 100 nM EGF. Cell suspensions were lysed with boiling 1% SDS and diluted with 9 volumes of an ice-cold lysis buffer (0.1 M HEPES, pH 7.3, 0.7 mM sodium orthovanadate, 10 mM *p*-nitrophenyl phosphate (PNPP), 10 mM EGTA, 5.5% Triton X-100, 0.5 M β-glycerophosphate, 10 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM ammonium molybdate, 12 mM DFP, 5 mg/ml leupeptin, 2 mg/ml aprotinin and 7 mg/ml pepstatin A). Cell lysates were incubated on ice for 20 min and then centrifuged for 5 min at $7000 \times g$. Supernatants were incubated overnight with the primary antibody, at a final concentration of 1 mg/ml, and with anti-rabbit (IgG, whole molecule) antibody conjugated to agarose beads (ratio agarose beads/cell lysates 1:2). Immune complexes were recovered by centrifugation. Pellets were washed two times with lysis buffer, two times with buffer A (0.1 M Tris-HCl, pH 7.4, 0.4 M LiCl) and two times with buffer B (0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 1 mM EDTA). Western blotting was performed as described previously [17].

2.4. MAP kinase enzymatic assay

The enzymatic activity of $p42^{mapk}$ was first measured in vitro as initially described [18]. 16 μM MBP was diluted in kinase buffer (13.4 mM HEPES, pH 7.3, 25 mM $MgCl_2$, 30 mM Na_2VO_3, 5 mM PNPP, 2 mM EGTA, 2 mM cAMP-dependent kinase inhibitor, 21 mCi [γ-^{32}P]ATP/ml (7 nM), and 68 mM unlabeled ATP). To initiate the phosphotransferase reaction, aliquots (20 μl) of kinase buffer were mixed 1:1 (v/v) with the cell lysates. The reaction was carried out at 25°C for 10 min, and terminated by blotting 20 μl of the reaction mixture onto ion-exchange cellulose phosphate filters. After washing, they were counted for radioactivity. The enzymatic activities of the mentioned kinases were also measured following the in-gel kinase assay as described previously [19]. Briefly, 250 μg/ml MBP was mixed with SDS-PAGE gel solution before gel polymerization. After electrophoresis, gels were washed free of SDS by incubation with 20% isopropanol in buffer A (100 mM Tris-HCl, pH 8, 5 mM β-mercaptoethanol). Gels were then incubated with buffer A containing 6 M guanidine-HCl and proteins were renatured by incubation at 4°C for 6 h in buffer A containing 0.04% Tween 40. Renatured gels were overlaid with kinase buffer (20 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 25 mCi [γ-^{32}P]ATP) and incubated at room temperature for 30 min. Gels were extensively washed with 1% pyrophosphate in 5% trichloroacetic acid, dried and exposed to X-ray films. For immunoprecipitation experiments cell lysates were obtained as described above except that the boiling 1% SDS step was omitted. Beads were resuspended in 30 μl of lysis buffer prior to electrophoresis loading.

3. Results

3.1. MAPK basal activity was increased in M-arrested cells

As indicated in Fig. 1A, an increased (up to 3.7-fold) MAPK activity was observed in interphase HeLa cells following incubation of cells with EGF. In contrast, a minimal activation was observed in M-arrested HeLa cells under similar conditions. Interestingly, the level of basal activity of these M-arrested cells was consistently higher than that of interphase cells in the absence of any stimulus. To explore these observations further, we first performed an immunoprecipitation with anti-$p42^{mapk}$ antibodies, to unequivocally confirm the kinase activity that been analyzed was that of the 42-kDa isoform of MAPK, and second a renatured in-gel kinase assay against the substrate embedded in the gel matrix. These experiments yielded confirmatory results (Fig. 1B): (i) an increase in phosphotransferase activity elicited by EGF most prominently in interphase cells, and (ii) an overall high basal activity level in mitosis.

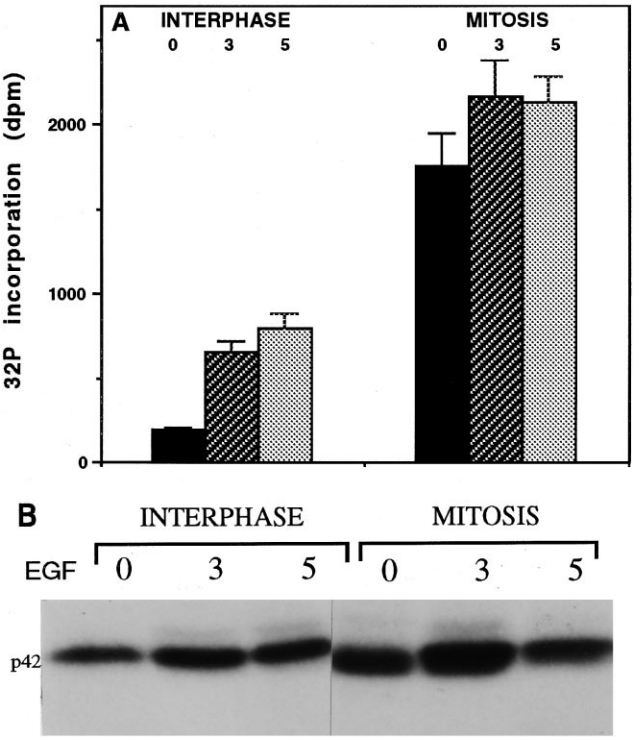

Fig. 1. Enzymatic analysis of MAPK in HeLa cells. A: In vitro kinase enzymatic activity analysis. Interphase and M-arrested HeLa cells (1×10^7 cells/ml) were treated with 100 nM EGF for 0, 3 or 5 min, and lysates (1.6 mg/ml protein) were obtained as indicated above. These were subjected to an in vitro kinase assay against MBP in the presence of [γ-^{32}P]ATP using the same total protein concentration (20 μg in 40 μl, final reaction volume, for each condition). Solid bars: controls; hatched bars: EGF-stimulated cells. The figure represents the mean ± S.E.M. of four experiments. B: Anti-$p42^{mapk}$ immunoprecipitates were electrophoresed in SDS polyacrylamide gels polymerized in the presence of 250 μg/ml MBP. After electrophoresis gels were subjected to the 'in-gel' kinase reaction involving protein renaturation and overlaying with [γ-^{32}P]ATP as explained in Section 2. Shown are regions of the gels between 33 and 48 kDa, representative of three other experiments performed separately.

3.2. MAPK was strongly tyrosyl phosphorylated in response to EGF in interphase cells

Since an increase in enzymatic activity might be the result of a covalent modification (i.e. phosphorylation) on the MAPK molecule, we checked for mobility shifts in SDS gels before and after EFG stimulation of HeLa cells. Interphase HeLa cells treated with EGF underwent mobility shifts in $p42^{mapk}$ shortly after stimulation (Fig. 2A). Note an upper band (*p42) present in the interphase stimulated cells that is absent in the mitotic counterpart, and a lower band (p42) present in mitotic cells at all times, and in interphase cells present only in resting conditions. Conversely, M-arrested cells failed to show any mobility shift, in agreement with the enzymatic assay data. The mitotic population failed to show any significant change even at the highest dose of ligand used (100 nM).

Immunoprecipitation of MAPK further demonstrated that a tyrosyl phosphorylated form of MAPK was elevated with EGF (Fig. 2B). Quantification of the *p42 band (OD × Area) expressed as a PY/MAPK ratio was: $[\text{Interphase}]_{-\text{EGF}} = 1.8/0.50 = 3.6$ $[\text{Interphase}]_{+\text{EGF}} = 5.7/0.36 = 15.8$, resulting in an activation by EGF of 4.4-fold. Conversely, no changes on ty-

rosyl phosphorylation were detected on the mitotic counterpart even though enough MAPK had been brought down by the antibody (Fig. 2C). Interestingly, little phosphotyrosine could be detected in M-arrested cells. Since mitotic cells were prepared by exposing unsynchronized cells to colcemid for 16 h, interphase controls were run with cells treated with the drug for only 1 h. The phosphorylation pattern of these cells is virtually identical to that derived from freshly harvested interphase cells (not shown) suggesting that the observed effects are not due to colcemid per se, but rather result from the cell cycle phase.

3.3. *The EGF•R and associated proteins were activated in M-arrested cells to a lower extent*

Exploring the differences in responsiveness towards EGF of the interphase and mitosis cells, we found that the lack of an increase on tyrosyl phosphorylation in M-arrested cells was not specific for $p42^{mapk}$, since other proteins were tyrosine phosphorylated to a lower extent in mitosis (Fig. 3A). Example of these were p183, p158 and p122. Since MAPK tyrosine phosphorylation did not take place in mitotic cells stimulated with EGF, we tested whether the EGF-induced signaling pathways were interrupted at the point of the receptor. As indicated in Fig. 3B, starting from equal protein concentrations on both subpopulations the anti-EGF•R antibody was able to immunoprecipitate the receptor (p183 protein) that was in the tyrosyl phosphorylated form in samples derived from EGF-treated cells. It could be observed that the phosphorylated receptor was 20–30% decreased in mitotic phase with respect to interphase. Similar results were obtained with SH-2 and SH-3 domain-rich proteins that associate with the EGF receptor, particularly PLCγ1 (Fig. 3C,D) and GAP (not shown). From dose-response experiments performed stimulating HeLa cells with 0.1–100 nM EGF, analysis of phosphorylated proteins and quantitative densitometric scans (OD×Area) of the EGF•R the results presented in Fig. 4A were obtained. It could be observed that although the apparent affinity of receptor activation was comparable in interphase and mitotic cells (~1 nM), saturation on mitosis never reached the levels of interphase. For comparison, Fig. 4B

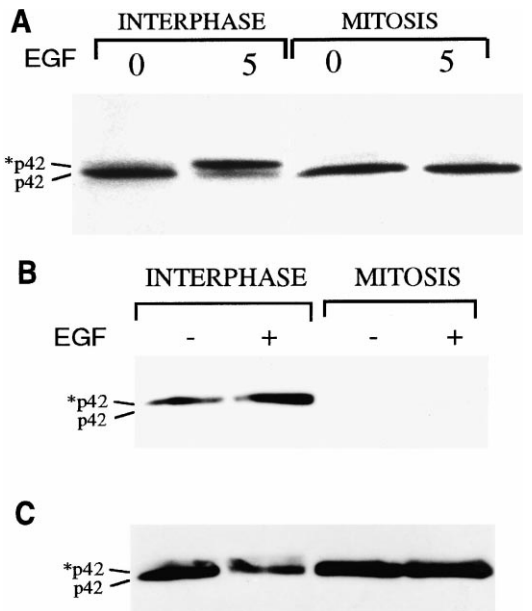

Fig. 2. Detection of p42 MAPK. A: In HeLa total cell lysates. HeLa interphase or mitotic cells (1×10^7 cells/ml) were incubated with or without 100 nM EGF for 5 min. The resulting blots were probed with anti-PY antibodies. Shown is a region of the blot that spans from 36 to 52 kDa. B: HeLa anti-$p42^{mapk}$ immunoprecipitates. MAPK was immunoprecipitated with anti-$p42^{mapk}$ antibody on SDS-boiled lysates (1.6 mg/ml protein) from interphase and mitotic HeLa cells unstimulated (−) or treated with 100 nM EGF (+). Western blots of immunocomplex beads were probed with anti-PY antibodies. C: HeLa anti-$p42^{mapk}$ immunoprecipitates and Western blots probed with anti-$p42^{mapk}$ antibodies. *p42 marks the position of the *phospho* form of p42-MAPK, while p42 marks the position of the *dephospho* form. Shown are representative experiments among five.

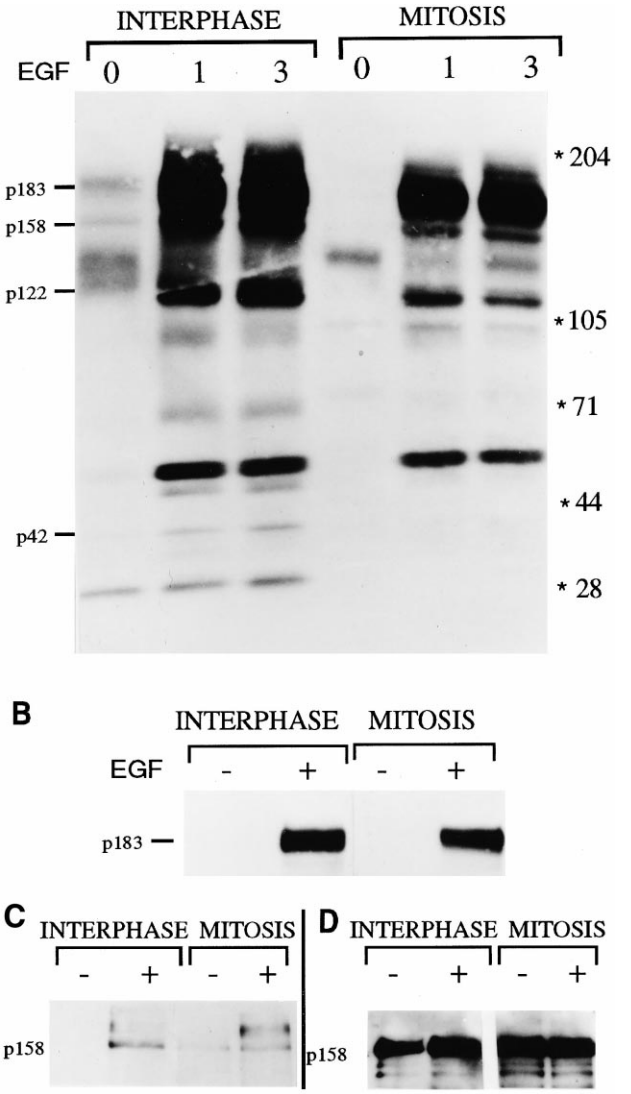

Fig. 3. A: Tyrosyl phosphorylation pattern of HeLa total cell extracts. Interphase or mitotic subpopulations were treated with or without EGF for 1 or 3 min. Lysates were subjected to Western blotting developed with anti-PY antibodies. The ticks on the left mark the position of relevant proteins as discussed in the text: p183: EGF•R; p158: PLCγ1; and p122: GAP. The asterisks on the right show the molecular weights (in kDa) of the protein markers run with the SDS gels. B–D: Signaling molecules upstream of MAPK. Lysates from interphase or mitotic subpopulations untreated or stimulated with EGF were immunoprecipitated with anti-EGF•R (B) or anti-PLCγ1 (C,D) and probed with anti-PY (B,C) or anti-PLCγ1 (D). Shown are results from a typical experiment among four.

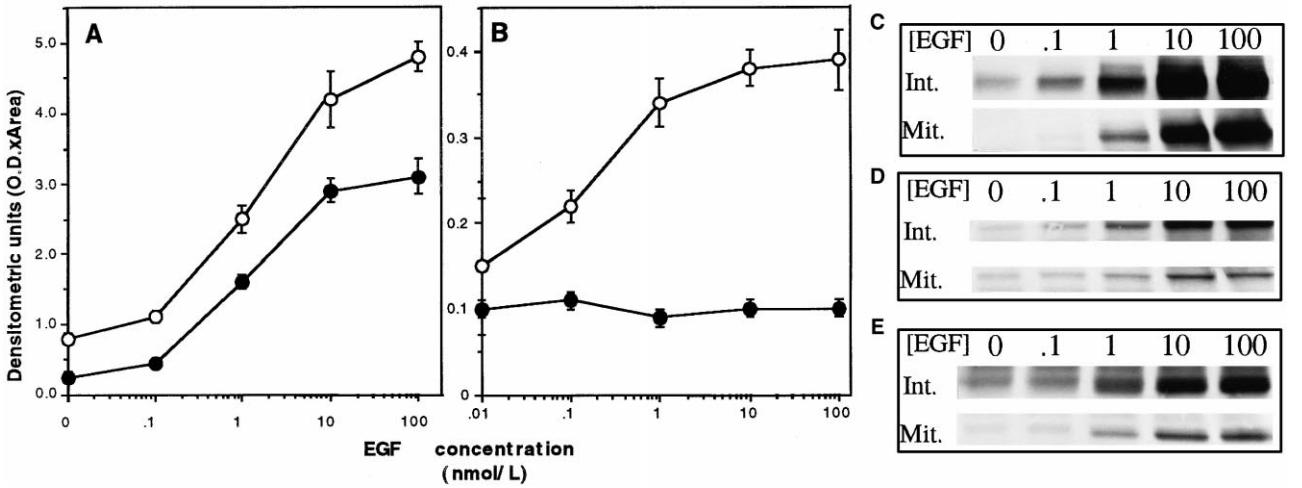

Fig. 4. Dose-response curve of the tyrosyl phosphorylated EGF•R and EGF•R. Interphase (○) or M-arrested (●) HeLa cells were incubated with the indicated concentrations of EGF (in nmol/ml) for 5 min and blots were generated. The p183/EGF•R (A) or the 42-kDa/MAPK (B) band in X-ray films was analyzed by quantitative densitometric scanning at 633 nm. The optical density (OD) was linear with the range considered. In the ordinate axis, the [OD×Area] of the band is presented. The figure represents the mean ± S.E.M. of four experiments. C–E: Blots developed with anti-PY of p183/EGF•R (from which scanning presented in A was obtained); p158/PLCγ1 and p122/GAP, respectively.

shows the scanning of the $p42^{mapk}$. Fig. 4C–E presents the phosphotyrosyl content of EGF•R, PLCγ1 and GAP. Thus, the small MAPK tyrosyl phosphorylation in mitosis in response to EGF seemed to stem from an early impairment of phosphorylation of the receptor and associated proteins.

4. Discussion

We provide evidence that (i) $p42^{mapk}$ becomes fully activated in response to EGF treatment in normally cycling (interphase) cells, but they fail to do so when cells are arrested in metaphase with colcemid treatment, and (ii) a higher level of basal activity exists in M-arrested cells as compared with the interphase counterpart. For the first finding, the fact that cells do not respond to EGF in mitosis, it could be advantageous to the mitotic cell. In the event of an external stimulation, the cell has the possibility of using all the kinases which have been activated upon entering mitosis. However, our results suggest that the mitotic cell does not take advantage of the mechanisms that have been described for other phases of the cell cycle (e.g. G_0/G_1 reentry that can account for part of the observed effects in interphase) upon external stimulation. Given that MAP kinase is a crucial signal transduction link, turning it on at inappropriate times could lead to a number of unwanted responses, such as the phosphorylation of MAPs that are already involved in the formation of the spindle. In this respect, MAP kinase that was exogenously added to *Xenopus* oocyte extracts in interphase induced a change to M phase of microtubule dynamics [20]. Microtubule protein p220 is a natural target for both MAP kinase and MPF also in *Xenopus* oocytes. Therefore, the mitotic cell suppresses conflicting signals while it is engaged in mitotic spindle formation, chromosome separation and all the events associated with this phase of the cell cycle.

As for the increase in MAPK activity in mitosis, it is known that during mitosis an elevated turnover of protein covalent modification (both Ser/Thr and Tyr phosphorylation) takes place. Only in the G_2/M transition, the regulatory light chain of myosin II, nuclear laminins, vimentin, and histones 1 and 3, are heavily phosphorylated. Our result of high MAPK enzymatic activity in cycling mammalian cells is in agreement with previous work in meiotic cell cycles of *Xenopus* oocytes [13,21] and with the activities of p38 MAPK and MPF in M-arrested fibroblasts [22]. Although it is clear that an exogenously activated MAPK causes profound changes during mitosis, it has been difficult to consistently show that MAPK is active in M-arrested cells. Edelmann et al. [23] have demonstrated that $p42^{mapk}/p44^{mapk}$ are already phosphorylated during anaphase of mitosis, thus making these two proteins active at the end of mitosis and through the very early part of G_1 after nocodazole release of 3T3 fibroblasts. We cannot exclude the possibility that in our studies some cells have progressed from metaphase to anaphase in the period from mechanical shake off of rounded mitotic cells, centrifugation, resuspension in fresh buffer and lysate preparation. Tamemoto et al. [24] have demonstrated that both p42 and p44 MAPKs are activated biphasically, in the G_1 phase and around the M phase in Chinese hamster ovary cells. Interestingly, there is little activity in nocodazole-arrested cells, but an elevated level of activity is found in the subsequent cell cycle after arrest release. Heider et al. [25] have reported a strong kinase activity, particularly in nocodazole-arrested HeLa cells, that slowly declines after drug removal. However, a failure of immunoprecipitate MBP kinase activity with anti-ERK2 antibodies led these authors to postulate the existence of a 40-kDa kinase as a novel mitotic kinase. In the report by Takenaka et al. [22], it is possible to observe that MAPK is activated in fibroblasts although at a lower rate than p38, and its study was not pursued any further. Often, results vary between arrested cells (by a spindle-disrupting drug) and their release into a subsequent cell cycle.

The surprising finding that activated MAPK in mitosis is not accompanied by tyrosyl phosphorylation can be explained in terms of a hyperphosphorylation of MAPK in Ser/Thr in this particular phase of the cycle and thus it could not be detected by the anti-PY antibodies. Indications that this might be the case are given by two recent reports (that also underline the importance of MAPK in somatic cell mitosis) in

which: (i) dephosphorylation by dual-specific phosphatase XCL100 of Erk2 renders *Xenopus* tadpole cells unable to remain arrested in mitosis after treatment with nocodazole [26] and (ii) dephosphorylation of phosphorylated Bcl-2 by the Ser/Thr kinase inhibitor staurosporine, but not the Tyr kinases inhibitor genistein, led to progression of M-arrested HeLa into interphase [27].

Going back to the observation of a lack of MAPK activation in response of mitotic cells to EGF, the most likely explanation could be found in terms of a failure of the mitotic EGF receptor to function normally in this phase of the cycle. Our data demonstrated that the EGF•R as well as two associated proteins, PLCγl and GAP, were tyrosyl phosphorylated to a lower extent in mitosis than they were in interphase. This is in agreement with [28]. Similarly, EGF•R tyrosine phosphorylation was not observed in rat-1 cells but it was in A331, MDAMB-468 and Her-5, cells that naturally or artificially overexpress the receptor [29]. Cells express the same number of receptors in any phase of the cycle. However, results are different in A431 and rat-1 cell lines, where binding is the same in G_0/G_1 and M in the former and different in the latter. Others have also found differences depending upon the method of synchronization. While EGF-induced activation of phospholipid hydrolysis did not operate properly in mitotic HeLa cells [30], phosphorylation of EGF•R and PLCγl is seen in mitosis after amethopterin inhibition and thymidine release, while little or no phosphorylation is seen in mitosis of nocodazole-arrested cells.

In the absence of a general consensus, it seems that a blockage such as that reported here, leading to compromised MAPK activation in response to EGF in mitosis, is initiated at the receptor level, but other components might still lie downstream of it, presumably the presence of an intricate system of proteases [31] and phosphatases that efficiently maintain the equilibrium of phosphorylated proteins [32].

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