Tetradecanoyl Phorbol Acetate-induced Microtubule Reorganization Is Required for Sustained Mitogen-activated Protein Kinase Activation and Morphological Differentiation of U937 Cells

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Abstract

Investigation of 12-tetradecanoyl phorbol 13-acetate (TPA)-resistant U937 cell clones has demonstrated that the normal sustained p42 mitogen-activated protein kinase (p42MAPK) activation produced by TPA treatment is absent. This is shown to be due to the inability of TPA to maintain activation of MAP/extracellular signal-regulated kinase kinase (MEK) and cRaf1. A direct relationship between sustained p42MAPK activation and differentiation is provided by the demonstration that blockade of MEK activation by PD098059 prevents TPA-induced morphological differentiation of wild type U937 cells. Using TPA-resistant clones, an involvement of microtubule reorganization and granule release is demonstrated by the ability of the microtubule depolymerizing agent nocodazole, to promote sustained p42MAPK activation in the presence of TPA. This response correlates with the lack of TPA-induced microtubule reorganization in these clones and the ability of nocodazole to partially bypass resistance to TPA. The results demonstrate a causal link between protein kinase C-dependent microtubule reorganization, sustained p42MAPK activation, and the induction of differentiation in U937 cells.

Introduction

The human monoblastoid cell line U937 has provided a useful cell culture model in the study of differentiation (1). Unlike the related, bipotential HL60 cell line, U937 cells differentiate exclusively toward macrophage-like cells, and among the agents that can trigger this differentiation are the phorbol ester class of PKC[3] agonists. Much evidence indicates that these agents do, indeed, act through PKC to trigger differentiation (reviewed in Ref. 2).

The PKC family itself comprises at least 11 mammalian gene products of which only the cPKC (α, β_1, β_2, γ) and nPKC (δ, ε, η, θ) isotypes are directly diacylglycerol/phorbol ester sensitive (3–6). The aPKC isotypes (ζ, ι) do not seem to respond to these agonists and, thus, may not mediate phorbol ester actions, although overexpression of PKCζ in U937 cells leads to an altered phenotype consistent with a higher probability of autonomous differentiation (7). U937 cells express PKCβ_1, β_2, δ, ε, and ζ (8), as well as a novel PKCη-related protein (9). It is presumed that activation of one or more of these is responsible for initiating the differentiation process, which is characterized by cell adherence, growth arrest and the up-regulation of macrophage markers (1, 2).

Previous studies from this laboratory have led to the isolation and characterization of a series of TPA resistant U937 cell lines (10). Detailed analysis of three of these resistant clones has demonstrated that although PKC expression and behavior is grossly normal and that certain acute responses remain intact, there is a subcellular "mis-localization" of PKCβ_2 in the three clones studied in detail (11). In U937 cells, this PKC isotype is associated with the microtubule network, and on stimulation with TPA there is a general reorganization of the microtubules. In the TPA-resistant cell lines, no reorganization of the microtubules occurs in response to TPA, correlating with the loss of PKCβ_2 from this compartment. Consequent to this defect, TPA does not induce an acute up-regulation of cell surface integrins derived from perinuclear granules. However, a partial reversal of the resistant phenotype can be elicited by nocodazole treatment, suggesting that the microtubule depolymerization and subsequent granule release play essential roles in the differentiation process triggered by TPA (11).

Further analysis of these TPA-resistant clones has revealed that unlike parental U937 cells, TPA does not induce a robust and sustained activation of p42MAPK. It is demonstrated here that the sustained activation of p42MAPK in U937 cells is dependent on microtubule reorganization and that it is essential for the TPA-dependent induction of morphological differentiation.

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[3] The abbreviations used are: PKC, protein kinase C; TPA, tetradecanoyl phorbol acetate; MAPK, mitogen-activated protein kinase; MEK, MAP/extracellular signal-regulated kinase kinase; MBP, myelin basic protein; WT, wild type; EGF, epidermal growth factor.

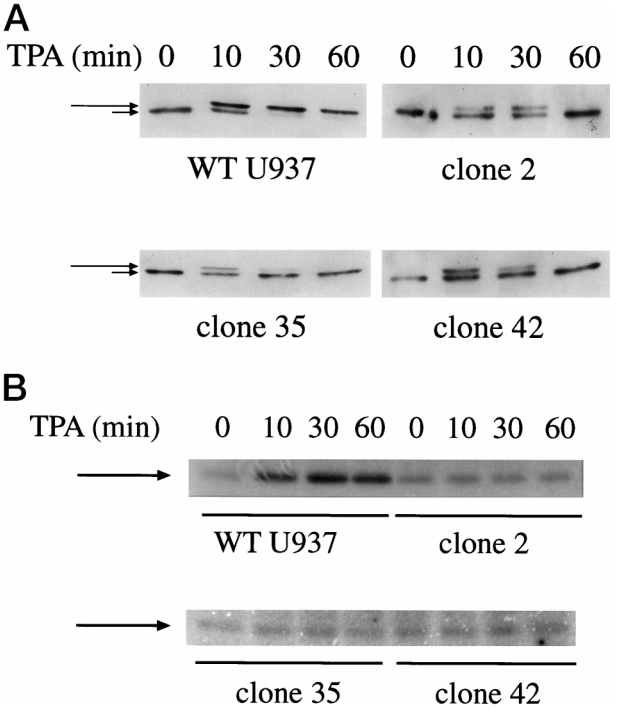

Fig. 1. Activation of p42MAPK in WT U937 cells and TPA-resistant clones. *A*, logarithmically growing WT U937 cells or TPA-resistant clones (2, 35, and 42) were exposed to TPA (10 nM) for 0, 10, 30, or 60 min, as indicated. The cells were then harvested, washed with PBS, extracted in SDS-PAGE sample buffer, and analyzed by Western blotting with pan-extracellular signal-regulated kinase antiserum. The inactive faster migrating (*short arrow*) and active (*long arrow*) forms of p42MAPK are indicated. *B*, cells were treated as in *A* and harvested directly into Laemmli sample buffer. In-gel kinase assays were performed using MBP as a substrate. Labeled protein is indicated by the *arrow.* Phosphorylation was quantified by scanning densitometry. The activation of p42MAPK in WT cells was 4.3-, 5.9-, and 5.2-fold for the 10-, 30-, and 60-min time points, respectively.

Results

Activation of p42MAPK in TPA-resistant U937 Cells Is Defective. In contrast to WT U937 cells, exposure of resistant clones to TPA (10 nM) does not result in differentiation, characterized by growth arrest and cell adherence. A number of acute TPA-induced events remain intact in these resistant cells (10, 11), however, examination of the time-dependent activation of p42MAPK in WT and resistant clones has revealed that the resistant clones display a more subtle defect. Acute exposure of WT U937 cells to TPA resulted in a time-dependent activation of p42MAPK, demonstrated by a shift in mobility on SDS-PAGE (Fig. 1*A*). Activation was nearly complete by 10 min and was sustained for at least 60 min. Although p42MAPK was expressed at comparable levels in both WT and resistant cells, TPA treatment of U937 clones 2, 35, and 42 resulted in only partial activation of p42MAPK, which was not sustained (Fig. 1*A*). For these resistant clones, the limited activation observed reached its peak at 10 min with no greater activation observed at shorter times (2–5 min; data not shown).

To confirm the activation of p42MAPK, in-gel kinase activities were determined. This demonstrated that WT cells, unlike the resistant clones produced a robust and sustained p42MAPK activation (Fig. 1*B*). In the WT U937 cells, activity was elevated ~6-fold over the basal level detected.

To investigate the apparent difference in p42MAPK activation in these TPA-resistant clones, we compared the activation and levels of expression of the upstream components of the p42MAPK cascade (*i.e.,* cRaf1 and MEK) in the resistant clone 2 and WT cells. Immunoblotting with a phospho-MEK antiserum (Fig. 2*A*) demonstrated that, in WT U937 cells, MEK was phosphorylated within 2 min and that this was sustained for at least 10 min. By contrast, clone 2 cells displayed a weak and transient phosphorylation of MEK in response to TPA. To confirm that the difference in MEK phosphorylation reflected a difference in activation, MEK was immunoprecipitated and assayed for p42MAPK kinase activity. After 10 min of treatment with 10 nM TPA, WT U937 cells showed a substantial activation of immunoprecipitated MEK (Fig. 2*B*); clone 2 cells showed no activation at this time point, consistent with the Western analysis above (see Fig. 2*A*). These differences in behavior of MEK activity and phosphorylation were not a function of expression, which was unaltered in WT and clone 2; similar levels of MEK expression were also observed in clones 35 and 42 (Fig. 2*B*, *inset*).

Consistent with the data described for MEK activation, the activation of cRaf1 was also found to contrast between WT U937 cells and clone 2 cells (Fig. 2*C*). After 10 min of TPA treatment, WT cells showed a ~2-fold activation of cRaf1; clone 2 cells showed no activation at this time, consistent with a lack of MEK activity (see above). No differences in the concentration of cRaf1 was noted for the different cell lines (Fig. 2*C*, *inset*).

Inhibition of MEK Activation by PD 098059 Blocks TPA-induced Differentiation. The lack of cRaf1/MEK/p42MAPK activation correlates with resistance in these TPA-resistant U937 cells and implicates this pathway in the TPA-induced morphological differentiation of U937 cells. To test this directly, we investigated the effects of the selective inhibitor of MEK activation PD098059 (12) on WT U937 cells after stimulation with TPA. WT cells were preincubated for 30 min in the presence or absence of PD098059 and then exposed to TPA for 2, 5, or 10 min. Fig. 3 shows that p42MAPK activation was completely inhibited by 30 μM PD098059 after TPA exposure. Consistent with published observations, *in vitro* PD098059 was found to block U937 cell immunoprecipitated cRaf1 phosphorylation of recombinant MEK (data not shown). Furthermore, although cRaf1 kinase activity was not increased in clone 2 cells exposed to TPA, its basal phosphorylation of MEK was also suppressed by the addition of PD098059 to an *in vitro* kinase assay (data not shown).

The ability of PD098059 to block cRaf1 activation of MEK and, hence, p42MAPK in U937 cells allowed investigation of the action of this inhibitor on TPA-induced differentiation. Continuous treatment of WT U937 cells with TPA resulted in both growth arrest and a delayed cell death (Fig. 4*A*). However, treatment with 30 μM PD098059, a concentration which proved neither cytotoxic nor cytostatic, blocked these effects of TPA. Thus, inhibition of the p42MAPK pathway blocks TPA-induced proliferation arrest in WT U937 cells.

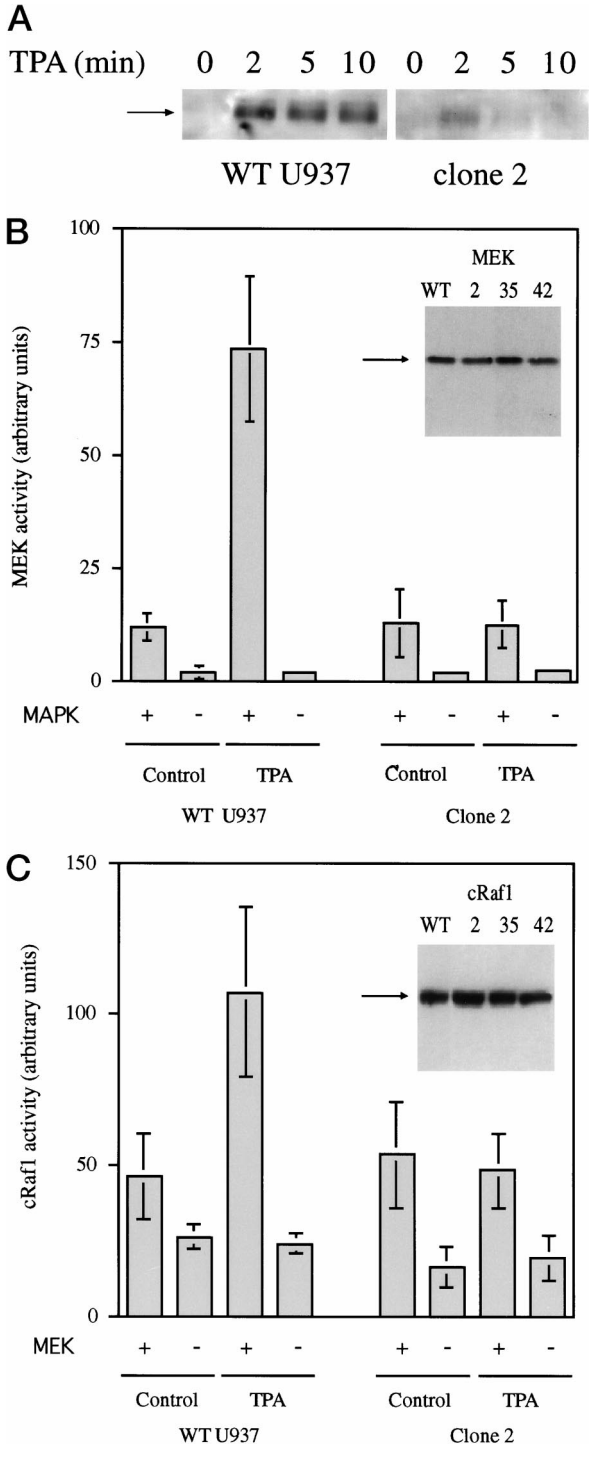

Fig. 2. Activation and expression of cRaf1 and MEK1 in WT U937 and TPA-resistant clones. *A*, MEK phosphorylation (*arrow*) was analyzed in WT U937 or clone 2 cells exposed to TPA (10 nM) for 0, 2, 5, or 10 min by Western blotting with phospho-MEK antiserum. MEK (*B*) and cRaf1 (*C*) activities in WT U937 and TPA-resistant clone 2 cells were determined by incorporation of ^{32}P into MBP in the presence or absence (*control*) of recombinant p42MAPK or p42MAPK and MEK, respectively, as described in "Materials and Methods." MEK or cRaf1 were immunoprecipitated from WT U937 cells and clone 2 whole cell lysates, prepared from untreated and TPA (10 nM for 10 min)-treated cultures. MEK1 (*B*, *inset*) or cRaf1 (*C*, *inset*) expression was analyzed in WT and TPA-resistant clones by Western blotting of whole cell lysates using specific monoclonal antisera.

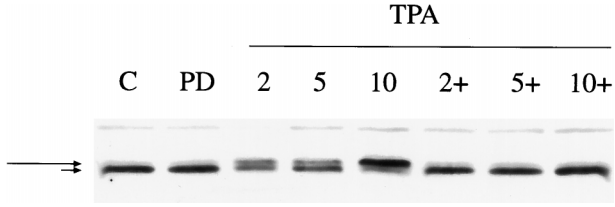

Fig. 3. Inhibition of p42MAPK activation by PD098059. Logarithmically growing WT U937 cells preincubated in the presence (+) or absence of 30 μM PD098059 for 60 min were cultured in the presence or absence of TPA (10 nM) for 5, 10, or 20 min. Extracts were analyzed by Western blotting as described previously. The inactive faster migrating p42MAPK (*short arrow*) and active (*long arrow*) forms of p42MAPK are indicated.

To assess the extent to which continuous p42MAPK activity is required for the observed growth arrest and adherence induced by TPA, WT cells were exposed to TPA and treated at various times with PD098059 (Fig. 4*B*). Pretreatment (30 min) or coincident treatment with the inhibitor completely blocked the TPA-induced adherence. However, progressive delay in PD098059 addition lead to a reduced inhibitory effect on adherence; on addition after 5 h, no effect was observed and, furthermore, there was no reversal of TPA-induced adherence.

In contrast to PD098059, the p38MAPK pathway inhibitor SB203580 (13) had no effect on TPA-induced U937 cell adherence and growth arrest. Thus, there seems to be a specific requirement for p42MAPK activation to commit the cells to both adherence and growth arrest. This commitment occurs during the first few hours of TPA exposure.

The Sustained Phase of p42MAPK Activation Is a Consequence of Microtubule Reorganization and Granule Release. We have shown previously that a central defect in these TPA-resistant clones is their inability to reorganize their microtubule networks; this correlates with a loss of PKCβ_2 from their microtubule compartments (11). To determine the relationship of this failure to control the microtubule network with the failure to sustain p42MAPK activation, use was made of the depolymerizing agent nocodazole, which, we have shown previously, will revert some of the resistant properties of the U937 cell clones (11). As shown in Fig. 5, nocodazole synergizes with TPA in the resistant clones to induce activation of p42MAPK. In the presence of nocodazole, the extent of TPA-induced activation at this 30-min time point is comparable with that observed for the WT cells. Nocodazole itself has no effect on p42MAPK activation in either U937 cells or any of the resistant clones. U937 cells display a characteristic perinuclear granule compartment that traffics to the cell surface on TPA treatment (11). This response is dependent on microtubule reorganization because in the TPA-resistant clones the granules fail to move in response to TPA (11). To determine whether granule movement correlated with the sustained activation of MAPK, we determined whether nocodazole was permissive for granule traffic in the TPA-resistant clones. In nocodazole-untreated cells, granules display a characteristic perinuclear location; treatment with nocodazole induces these granules to relocate to the cell surface (Fig. 6). This granule movement is not itself sufficient to cause p42MAPK activation, which requires

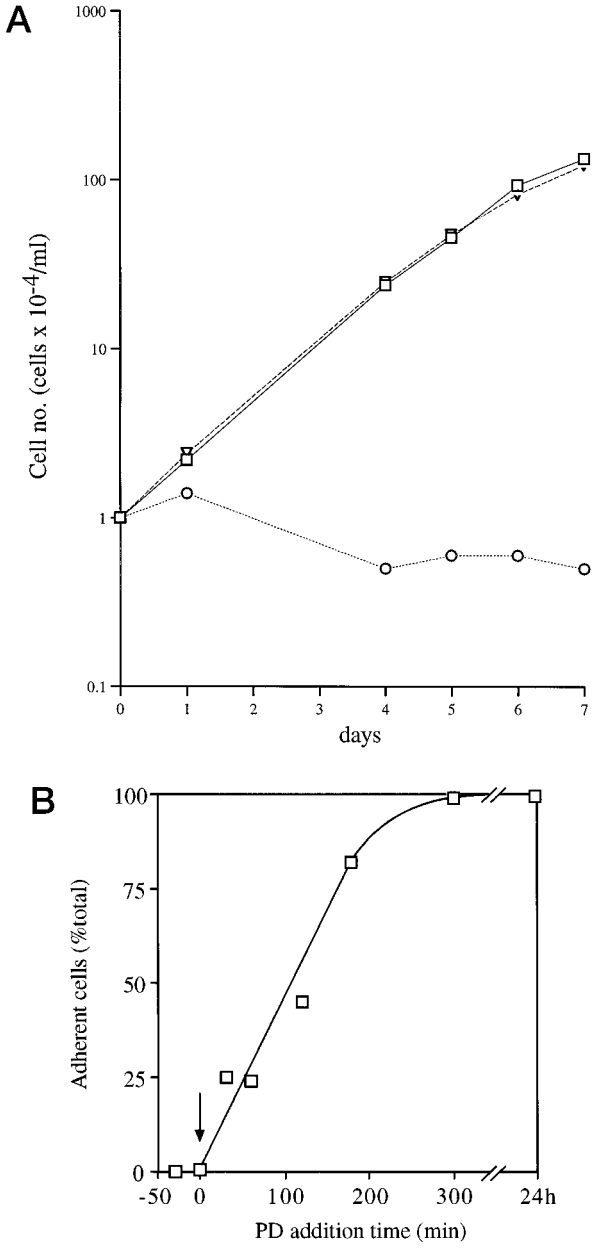

Fig. 4. MAPK activation is required for TPA-induced adherence and growth arrest in U937 cells. *A*, U937 cells were grown with no treatment (□), in the presence of 10 nM TPA (○) or in the presence of both 30 μM PD098059 and 10 nM TPA (▽). Treatment with 30 μM PD098059 alone had no effect on the growth of the cells (data not shown). Cell numbers were determined using a hemocytometer. *B*, U937 cells were treated with TPA (10 nM) at time 0 min (*arrow*). Cells were additionally treated with 30 μM PD098059 at the times indicated. Adherent cells were harvested and counted.

coincident TPA treatment (as described above); a further TPA-dependent step must be involved.

Discussion

The results presented here demonstrate that activation of the p42MAPK pathway is necessary for TPA-induced differentiation of U937 cells. This is evidenced both through study of

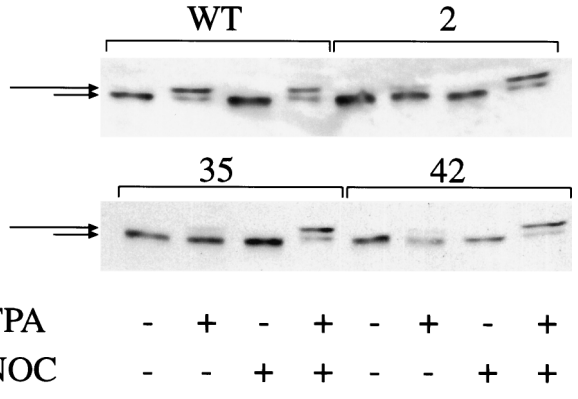

Fig. 5. Depolymerization of microtubules bypasses the block to p42MAPK induction in TPA-resistant cells. p42MAPK activation was analyzed in WT cells and TPA-resistant clones 2, 35, and 42. Cells were pretreated with nocodazole (NOC; 10 μg/ml for 10 min) before stimulation with TPA (10 nM for 30 min). Extracts were prepared and subjected to Western blotting. Active (slow migrating) and inactive p42MAPK species are indicated.

TPA-resistant clones, which display a defective TPA-induced p42MAPK response, and also by demonstration that the p42MAPK pathway inhibitor PD098059 blocks TPA-induced differentiation of WT U937 cells. It is further established that sustained activation of the p42MAPK pathway is a consequence of the reorganization of the microtubule network and the granule traffic normally observed in response to TPA. This is consistent with the notion that the induced granule release permits the action of a TPA-dependent, autocrine signal that is responsible for eliciting sustained p42MAPK activation.

Previous studies from this laboratory have led to the isolation and characterization of a series of clonal U937 cell lines that, unlike their parental counterpart, do not become adherent nor growth arrest in response to TPA stimulation (10). Interestingly, the resistance to TPA of these clones is manifested in only one part of the overall differentiation program. Thus, although resistant cells remain in cycle, fail to up-regulate integrins, and do not become adherent, TPA treatment still induces up-regulation of certain differentiated macrophage antigens (*e.g.,* p47phox; Ref. 10). These resistant clones, thus, define two apparently independent elements of the differentiation program. The finding here that the sustained activation of p42MAPK is defective in these resistant lines and that this is necessary for adherence and growth arrest in WT cells, places the p42MAPK cascade on the pathway to integrin up-regulation/adherence/growth arrest in U937 cells. Similarly it is concluded that sustained activation is irrelevant to the induction of p47phox. The separation of p47phox induction and adherence/growth arrest suggests that there may be a particular relationship between adherence and growth arrest. However, growth arrest, *per se,* is not sufficient to cause cell adherence because rapamycin will also arrest proliferation, but does not cause cells to adhere (data not shown).

The requirement for sustained activation of p42MAPK in U937 cell morphological differentiation has a parallel in PC12 cells, where nerve growth factor similarly induces a pro-

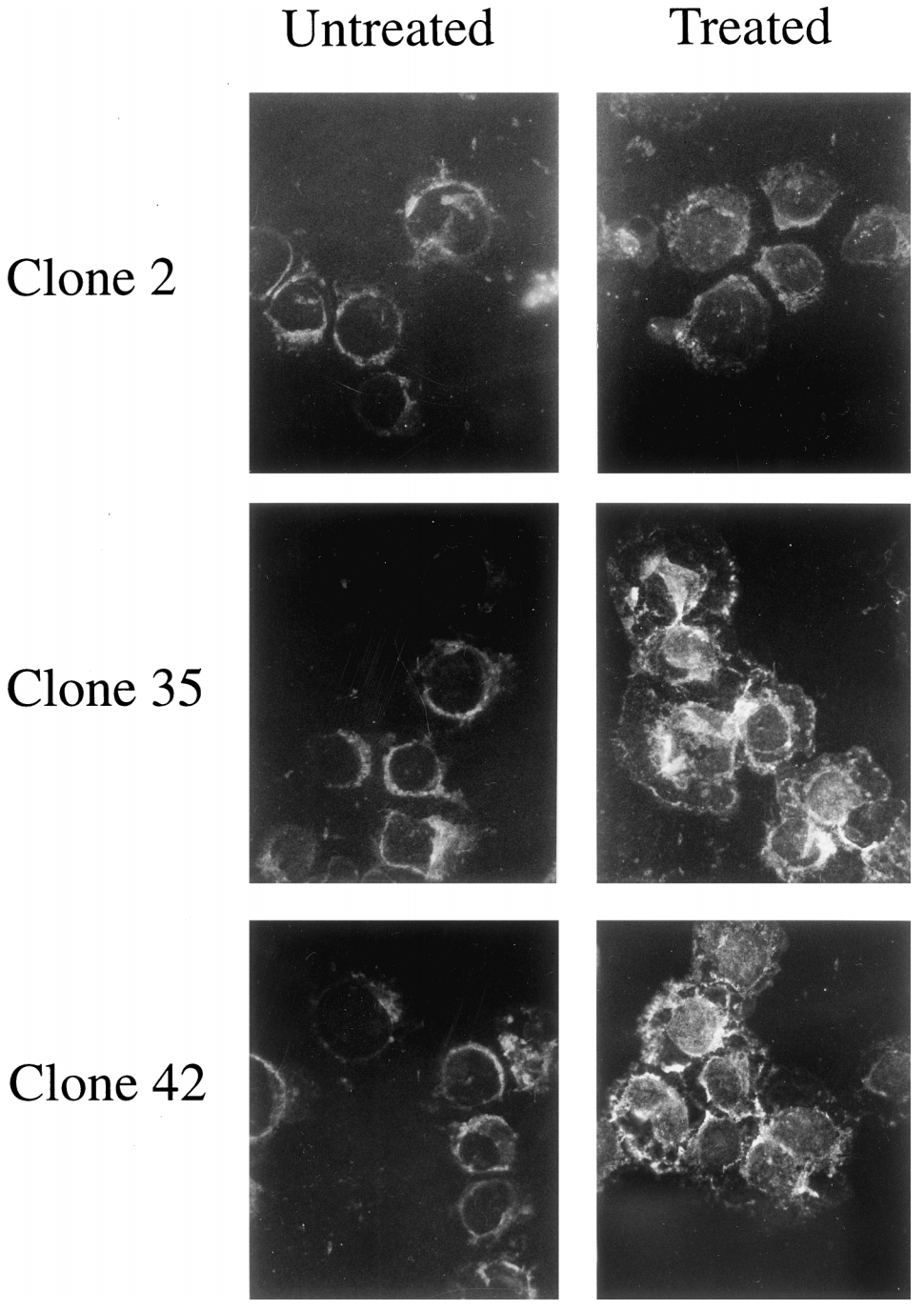

Fig. 6. Nocodazole induces granule traffic to the plasma membrane. U937 cell clones (as indicated) were treated with nocodazole for 2 min. Cells were fixed and stained for CD11b as described previously (zref11). In control untreated cells (*left*) the antibody detects perinuclear vesicles. After treatment with nocodazole (*right*), immunoreactivity is distributed throughout the cytoplasm with strong signals at the plasma membrane.

longed activation that seems essential for neurite outgrowth (14). Mitogens such as EGF induce only transient p42MAPK activation in PC12 cells and do not elicit neurite outgrowth (14). However, on EGF receptor overexpression, a sustained response to EGF can be obtained, which then induces neurite outgrowth (15); this highlights the importance of the dynamics of p42MAPK responses. Sustained p42MAPK activation in response to TPA has been shown also to be required for K562 cell differentiation (16, 17), but not for HL60 differentiation (18). Similar distinctions are apparent for other cell types/agonists; for example, retinoic acid-induced differentiation of HL60 cells does require p42MAPK activation (19). It is notable in the studies described here for U937 cells that the sustained MAPK activation is required for no more than 5 h to commit 100% of cells to become adherent.

The mechanism of TPA-induced p42MAPK activation is of particular interest. It has been demonstrated in various contexts that different PKC isotypes can induce the activation of p42MAPK (*e.g.,* Ref. 20 and see Ref. 21). This may occur through the direct regulation of cRaf1 (22, 23), although we

have noted that this link may be rather more complex (24). The finding here that the mechanism of sustained activation is consequential to the reorganization of the microtubule network with the subsequent delivery of integrin-containing granules to the cell surface is suggestive of an autocrine mechanism. Because nocodazole alone is unable to induce a p42MAPK response, any autocrine mechanism would still require a TPA-dependent step. However, conditioned medium from TPA-treated WT U937 cells does not induce differentiation of resistant lines, indicating that any autocrine effect is not due to release of soluble factors (data not shown). It is possible that the autocrine effect is membrane-limited. For example, the acute integrin up-regulation on the cell surface may be responsible for transducing this signal, and this itself may be dependent on the ability of TPA to modulate integrin function (25). It is notable that, in fibroblasts, a significant proportion of the p42MAPK is present in the microtubule compartment (26) and can influence cytoskeleton organization (27). Although this might extrapolate to a more direct relationship between the TPA-induced reorganization of the microtubules and p42MAPK in U937 cells, our preliminary data indicates that it is the granule release that is critical to the sustained activation; the microtubule reorganization is simply required to permit the granule release.

In conclusion, it is demonstrated that a specific defect associated with the mislocation of PKCβ_2 is the loss of sustained p42MAPK activation. This response is shown to be essential for TPA-induced morphological differentiation in WT U937 cells and could be triggered by TPA in combination with nocodazole in TPA-resistant U937 cells, a treatment known to bypass the differentiation block (11). The precise nature of the signal consequent to microtubule reorganization and membrane traffic remains to be determined.

Materials and Methods

Cell Culture. U937 promonocytic leukemia cells were cultured in RPMI 1640 at 37°C in a 5% CO_2 atmosphere. Derivation and partial characterization of TPA-insensitive clones designated 2, 35, and 42 have been described earlier (10). Cell density was maintained between 0.1 and 1 $\times$ 10^6 cells/ml in RPMI supplemented with 10% FCS containing 1000 units/ml penicillin and 100 μg/ml streptomycin.

As indicated in the text or figure legends, U937 cells were plated at 2 $\times$ 10^4/ml in RPMI containing 10% FCS and treated with the MEK activation antagonist PD098059 (generously provided by Dr. A. Saltiel, Parke-Davies). Cell number was evaluated using a hemocytometer. For immunofluorescence studies, cells were processed and stained as described previously (8).

Cell Lysis and Western Blot Analysis. U937 cells were harvested, washed in PBS, and lysed in 100 μl of Laemmli sample buffer/1 $\times$ 10^6 cells, heated at 95°C for 5 min, centrifuged at 12,500 $\times$ *g* for 5 min, and supernatants were collected for SDS-PAGE (28). Samples prepared in Laemmli buffer were analyzed by electrophoresis on 10% polyacrylamide SDS-PAGE gels. Gels were transferred onto nitrocellulose and subjected to immunoblotting using polyclonal rabbit or mouse monoclonal anti-cRaf1, anti-MEK 1 and 2, antiphospho-MEK, and pan-extracellular signal-regulated kinase antibodies (monoclonal antibodies were obtained from Transduction Laboratories, Affiniti Research Products Ltd., Nottingham, United Kingdom). The secondary (peroxidase-linked donkey antirabbit or antimouse) antibodies, as well as the enhanced chemiluminescence Western blotting detection system, were from Amersham.

Immunoprecipitation. cRaf1 and MEK were immunoprecipitated from U937 lysates prepared from cells cultured in the presence or absence of TPA (10 nM) or the ethanol vehicle (0.1% final). Lysis medium contained 50 mM Tris-HCl (pH7.5), 100 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 50 μg/ml PMSF, 10 mM benzamidine, 125 μg/ml aprotinin, 250 μg/ml leupeptin, and 1 μM microcystin. Samples were precleared with insoluble protein A (1% final concentration at 4°C for 15 min) and centrifuged at 12,500 $\times$ *g* for 1 min. Supernatants were tumbled for 4 h with extracellular protein A agarose precoupled to polyclonal cRaf1 or MEK antisera. Antibody-coupled protein A beads were washed in lysis buffer, PBS, and finally resuspended in kinase reaction buffer (25 mM HEPES, 10 mM para-nitrophenylphosphate, 0.1 mM EDTA, 1 μM microcystin, and 10 mM $MgCl_2$).

***In Vitro* Kinase Assay.** cRaf1 and MEK activities immunoprecipitated from U937 cells were determined by incorporation of ^{32}P into MBP. cRaf1 kinase activities were determined using a coupled assay, in the presence of recombinant p42MAPK (10 μg) and MEK (0.5 μg). Similarly, MEK activity was assayed in the presence of recombinant p42MAPK. Assays used 20 μl of protein A beads retaining immunocomplexes of cRaf1 or MEK and were carried out in kinase reaction buffer (see above). After incubation for 12 min at 30°C, 10 μM [γ-^{32}P]ATP and 40 μg of MBP were added and incubated for an additional 12 min when 20 μl of the mixture was removed and spotted on P81 phosphocellulose filters (Whatman). Filters were washed three times in 30% acetic acid and ^{32}P-phosphate incorporation was determined. Nonspecific activity was determined by exclusion of the proximal kinase substrate (see figures), and basal activities were determined in the absence of immunocomplex.

Activation of MEK was also determined by Western blotting with an antiserum specific for the active phosphorylated from of the protein (see above). In addition to Western blotting, MAPK activity was determined by in-gel kinase assay using MBP (0.5 mg/ml) as a substrate, according to the manufacturer's recommended procedures (Stratagene).

Acknowledgments

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