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Defective Regulation of Mitogen-Activated Protein Kinase Activity in a 3T3 Cell Variant Mitogenically Nonresponsive to Tetradecanoyl Phorbol Acetate

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Mitogen-activated protein (MAP) kinase is a serine/threonine-specific protein kinase which is activated in response to various mitogenic agonists (e.g., epidermal growth factor, insulin, and the tumor promoter tetradecanoyl phorbol acetate [TPA]) and requires both threonine and tyrosine phosphorylation for activity. This enzyme has recently been shown to be identical or closely related to pp42, a protein which becomes tyrosine phosphorylated in response to mitogenic stimulation. Neither the kinases which regulate MAP kinase/pp42 nor the in vivo substrates for this enzyme are known. Because MAP kinase is activated and phosphorylated in response both to agents which stimulate tyrosine kinase receptors and to agents which stimulate protein kinase C, a serine/threonine kinase, we have examined the regulation and phosphorylation of this enzyme in 3T3-TNR9 cells, a variant cell line partially defective in protein kinase C-mediated signalling. In this communication, we show that in the 3T3-TNR9 variant cell line, TPA does not cause the characteristically rapid phosphorylation of pp42 or the activation and phosphorylation of MAP kinase. This defective response is not due to the absence of the MAP kinase/pp42 protein itself because both tyrosine phosphorylation of MAP kinase/pp42 and its enzymatic activation could be induced by platelet-derived growth factor in the 3T3-TNR9 cells. Thus, the defect in these variant cells apparently resides in some aspect of the regulation of MAP kinase phosphorylation. Since the 3T3-TNR9 cells are also defective with respect to the TPA-induced increase in ribosomal protein S6 kinase, these in vivo results reinforce the earlier in vitro finding that MAP kinase can regulate S6 kinase activity. These findings suggest a key role for MAP kinase in a kinase cascade involved in the control of cell proliferation.

Activation of tyrosine kinase transmembrane receptors results in stimulation of a diverse array of cellular activities, such as glycolysis, protein synthesis, and transcription. However, in most cases in which the regulation of these cellular activities has been adequately investigated, critical phosphorylations of regulatory proteins occur not only on tyrosine but on serine and threonine as well. Thus, it seems likely that "switch kinases", which are regulated by tyrosine phosphorylation but function as serine/threonine kinases, are pivotal elements in the hormonal control of growth and metabolism. Elucidation of the pathways by which cell surface receptors control growth and metabolism will require analysis of the regulation of these switch kinases and determination of their downstream substrates.

Mitogen-activated protein (MAP) kinase was the first switch kinase to be so identified (35). This enzyme was originally described by Ray and Sturgill (34) as a serine/threonine-specific protein kinase which was activated by treatment of 3T3-L1 cells with insulin and which could be partially purified by sequential DEAE and hydrophobic-interaction chromatography (36). Subsequently, it was found that this enzymatic activity could be stimulated by various mitogenic agents, including the tyrosine kinase agonists epidermal growth factor (EGF) (23, 37) and platelet-derived growth factor (PDGF) (this article), as well as the tumor promoter tetradecanoyl phorbol acetate (TPA) (23, 37), whose receptor is the serine/threonine protein kinase, protein kinase C (PK-C). That MAP kinase activity can be stimulated either by tyrosine kinase agonists or by serine/threonine kinase agonists suggests that this enzyme functions in vivo to integrate physiological responses involving both tyrosine and serine/threonine phosphorylations. This suggestion is supported by the finding that MAP kinase must be phosphorylated on both threonine and tyrosine in order to be active (2).

Recently, MAP kinase was shown to be identical or closely related to pp42, a cellular protein known to become phosphorylated on tyrosine in response to various mitogens including EGF, PDGF, and TPA (24, 37). The dual control over pp42 phosphorylation by both tyrosine phosphorylation and PK-C was demonstrated not only by the fact that TPA could stimulate tyrosine phosphorylation of this protein (6, 16, 21) but also by the fact that down-modulation of PK-C by chronic TPA treatment could blunt the ability of EGF (42), PDGF (26), or serum (19) to stimulate this tyrosine phosphorylation. We have found that the activation of MAP kinase activity in response to EGF displays a similar partial dependence on cellular PK-C (37a), and evidence that this is also true for the case of serum stimulation has been presented by Ferrell and Martin (19). However, the proximal kinases which phosphorylate MAP kinase remain unidentified.

The important in vivo substrates for MAP kinase also have not been identified with certainty. MAP kinase can phosphorylate a variety of in vitro substrates, including microtubule-associated protein 2 (23, 32, 34), myelin basic protein (1, 17), and ribosomal protein S6 kinase II (22, 39). All of these substrate proteins are known to become phosphorylated in vivo (3, 31, 33, 41), but it is uncertain whether MAP

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kinase is the enzyme responsible for these in vivo phosphorylations. Only in the case of S6 kinase-II has phosphorylation by MAP kinase been shown to have functional consequences. This ribosomal protein kinase, when inactivated by phosphatase treatment, can be partially reactivated by phosphorylation with MAP kinase (22, 39). Thus, one of the in vivo functions of MAP kinase may be in the regulation of protein synthesis.

To gain insight into the regulation and function of MAP kinase we have made use of 3T3-TNR9 cells, a variant derived from the Swiss 3T3 line on the basis of its mitogenic nonresponsiveness to TPA (11). These cells have normal levels of PK-C and display many of the early phosphorylation and metabolic responses to TPA treatment (7, 12, 18) which occur in the parental 3T3 cells. However, unlike the parental 3T3 cells, they do not show an increase in ornithine decarboxylase activity or DNA synthesis in response to TPA (11, 12). Thus, they appear to be defective in some aspect of PK-C-mediated intracellular signalling. Because of the expected role of MAP kinase in integrating tyrosine kinase and PK-C-stimulated signals, we felt that this was an appropriate line to use for these investigations.

In this article, we show that rapid TPA-induced phosphorylation and activation of MAP kinase/pp42 are defective in 3T3-TNR9 cells. On the other hand, PDGF is able to stimulate both tyrosine phosphorylation and enzymatic activation of MAP kinase in the 3T3-TNR9 cells, indicating that these cells do not lack MAP kinase but that their defect resides in the regulation of this kinase. The variant cells have also been shown to be defective in the TPA-induced increase in S6 kinase activity (18). Thus, our results provide in vivo support for the in vitro finding that MAP kinase regulates the activity of S6 kinase and raise the possibility that this kinase cascade is important for mitogenic stimulation.

MATERIALS AND METHODS

Materials. TPA was purchased from Sigma Chemical Co., St. Louis, Mo.; PDGF was purchased from Collaborative Research, Inc., Bedford, Mass.; DE52 was purchased from Whatman Biosystems Ltd., Maidstone, England; phenyl Superose column from Pharmacia; Ampholines were purchased from LKB; Tween 20 was purchased from Bio-Rad, Richmond, Calif.; bovine serum albumin (BSA) fraction V was purchased from Boehringer Mannheim; and fetal calf serum was purchased from GIBCO, Grand Island, N.Y. [^{125}I]labeled protein A and $^{32}P_i$ were purchased from Amersham, Arlington Heights, Ill. Specific antiphosphotyrosine antibodies were generated and affinity purified in this laboratory as described previously (37).

Cell culture. Swiss 3T3 and TNR9 cells were cultivated at 37°C in a humidified atmosphere of 7.5% CO_2 with Dulbecco modified Eagle medium supplemented with 10% fetal calf serum until confluence. Cells were never passaged more than 15 times before being discarded. Unless specified, 2-day-confluent cells were starved for 2 h in fetal calf serum-deprived Dulbecco modified Eagle medium before activation.

Immunoblotting. Whole-cell lysates (typically 100 to 150 μg of protein per lane) or purified material (1 to 3 mg of protein per fast protein liquid chromatography [FPLC] run) were electrophoresed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels at 25 mA for 4 h and electroblotted overnight onto nitrocellulose (BA83; Schleicher & Schuell) in 25 mM Tris–192 mM glycine–20% methanol–0.03% SDS. Ponceau S-stained filters were preblocked in 3% BSA–0.15 M NaCl–50 mM Tris (pH 7.4)–0.5% Nonidet P-40–0.1% Tween 20 before addition of affinity-purified antiphosphotyrosine antibodies at 4 to 8 μg/ml for 3 h. Filters were washed, probed with [^{125}I]protein A (1 μCi/ml), washed again, and autoradiographed on Kodak X-Omat RP film.

Two-dimensional gel electrophoresis. Cells in 100-mm-diameter dishes (0.2 to 0.5 mg of protein per dish) were starved in serum-deprived, phosphate-free Dulbecco modified Eagle medium for 1 h before the addition of $^{32}P_i$ at 3 mCi/ml for 2 h. Cell lysates were prepared as previously described (37). Two-dimensional (2-D) gel electrophoresis followed the methods of Garrels (20). All 2-D gel samples were loaded in the first dimension onto isoelectric focusing gels containing a 1:2:2 mixture of pH 3.5 to 10, 5 to 7, and 6 to 8 Ampholines. The resolving gels were SDS–10% polyacrylamide gels for the second dimension. Proteins were blotted onto Immobilon membranes (Millipore) as described earlier in this paper except that the blotting buffer was without SDS. Blots were then treated with 1 M KOH at 56°C for 2 h. Autoradiograms were performed on Kodak XAR films.

Phosphoamino acid analysis of blotted proteins was essentially as described previously (15, 25).

Purification of MAP kinase/pp42. The MAP kinase/pp42 protein was partially purified by sequential DEAE-cellulose and phenyl Superose chromatography and assayed for its activity according to the procedures of Ray and Sturgill (35, 36). Briefly, a fraction containing the MAP kinase activity present in nucleus-free cell lysates was eluted from DE52 at 350 mM NaCl and loaded onto a phenyl Superose column (1 to 3 mg of protein per run). In a 0 to 60% linear gradient of ethylene glycol, the peak of MAP kinase activity is typically recovered at 37% ethylene glycol as assessed by the in vitro phosphorylation of microtubule-associated protein 2. Microtubule-associated protein 2 was purified from bovine brain by the method of Kim et al. (27).

Aliquots (one-third each) of fractions eluted through the phenyl Superose step were precipitated as described by Bensadoun and Weinstein (4) with deoxycholate-trichloroacetic acid and reconstituted with hot SDS sample buffer before electrophoresis and phosphotyrosine antibody immunoblotting.

RESULTS

Phosphorylation of pp42. Acute TPA treatment (300 ng/ml) rapidly induced tyrosine phosphorylation of a 42-kDa protein in parental Swiss 3T3 but not in 3T3-TNR9 cells, as detected by immunoblotting with antiphosphotyrosine antibodies of whole-cell extracts separated by gel electrophoresis (Fig. 1A). We previously have identified this tyrosine-phosphorylated protein as the pp42 protein and have described its relatedness to MAP kinase (37).

The absence of rapid phosphorylation of pp42 in TPA-treated 3T3-TNR9 cells is specific to this agent since PDGF was able to induce this phosphorylation in both the parental and the variant cell lines (Fig. 1B). In addition, the time courses for PDGF-induced phosphorylation of pp42 were very similar over this period in 3T3 and in TNR9 cells, although the magnitude of the phosphorylation was generally more pronounced in the parental cell line. EGF also induced pp42 tyrosine phosphorylation in the TNR9 cells to a lesser extent than in the 3T3 cells (data not shown). This blunted phosphorylation of pp42 in the TNR9 cells treated with peptide mitogens presumably reflects the fact that this phosphorylation is partially dependent on PK-C even when the stimulating agent is PDGF or EGF (26, 42). These results

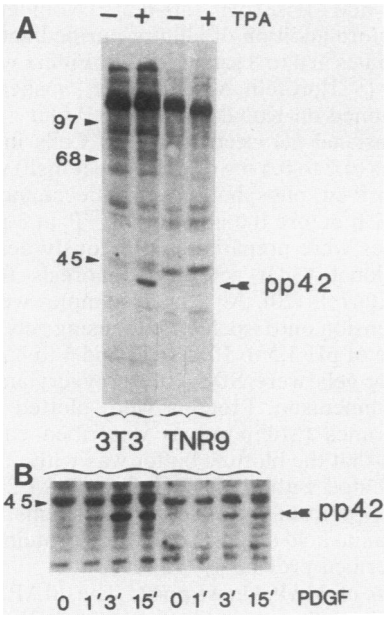

FIG. 1. Immunoblotting of whole cell lysates from Swiss 3T3 and TNR9 cells with antiphosphotyrosine antibodies. Serum-starved cells were treated with TPA (300 ng/ml for 10 min) (A) or with PDGF (10 ng/ml for 1, 3, and 15 min) (B). Immunoblots of 3T3 cell lysates are displayed in the left half of each panel, and immunoblots of TNR9 cell lysates are in the right half of each panel. A total of 120 μg of protein per lane was loaded for each cell line. The results are characteristic of more than four independent experiments. Autoradiography of the immunoblot of the PDGF-stimulated cells represents an overexposure, so as to reveal the phosphorylation at the 1-min time point. Numbers on the left are molecular masses (in kilodaltons).

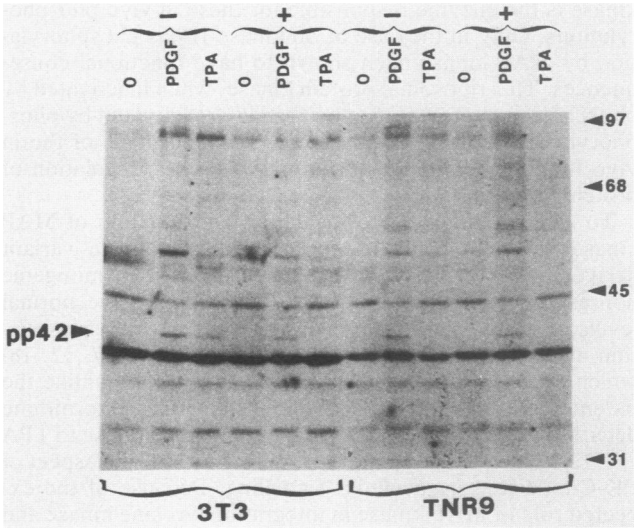

FIG. 2. Effect of chronic TPA treatment of 3T3 and TNR9 cells on pp42 tyrosine phosphorylation. Confluent cells were (+) or were not (−) pretreated for 26 h with 1 μg of TPA containing 1% serum per ml before a 10-min addition of PDGF (10 ng/ml) or TPA (300 ng/ml). Antiphosphotyrosine immunoblots of whole-cell lysates are shown. For each cell line, 90 μg of protein per lane was loaded the position of the pp42 protein is indicated. Numbers on the right are molecular masses (in kilodaltons).

indicate that pp42 is present in the TNR9 cells but that the portion of its tyrosine phosphorylation which is dependent on PK-C signalling is defective.

To determine whether the TNR9 cells were specifically defective in the PK-C-dependent component of pp42 stimulation, we determined the effects of chronic TPA treatment on the ability of PDGF to stimulate pp42 phosphorylation in these cells. Chronic treatment with TPA prevents any further TPA-induced change in pp42 tyrosine phosphorylation, as expected if PK-C has been down-modulated in these cells (Fig. 2). However, although induction of pp42 phosphorylation by PDGF was reduced about twofold by chronic TPA treatment of the 3T3 cells, which is in agreement with previous results (19, 26, 42), PDGF-induced phosphorylation of pp42 was not changed by TPA treatment of the TNR9 cells (Fig. 2). (A protein of approximately 55 kDa whose tyrosine phosphorylation is coregulated with pp42 is also revealed in these immunoblots. The identity of this protein is not known.) These results support the idea that in TNR9 cells the PK-C-independent portion of the pp42 phosphorylation pathway is still functional, even though the PK-C-dependent component is defective. However, since pp42 in these experiments is resolved only on 1-D gels, it is also possible that the tyrosine-phosphorylated band we observe has two components which are regulated by different pathways.

The results in Fig. 1, which show an absence of TPA-induced pp42 tyrosine phosphorylation in TNR9 cells, are in apparent conflict with our earlier conclusion (7), based on different methods, that TPA could induce an increase in tyrosine phosphorylation on proteins of 42 to 45 kDa in the TNR9 cell line. Initially we suspected that differences in cell culture conditions could explain this discrepancy. In the work described here, cells were stepped down in serum-free medium prior to stimulation, whereas in the previous work, cells were stimulated in conditioned medium containing serum factors. However, we have repeated the TPA stimulation of 3T3-TNR9 cells in the presence of conditioned medium and still find that these cells do not display pp42 phosphorylation, as detected with Western immunoblotting (data not shown). Thus, cell culture conditions are not responsible for the differences with our earlier work.

The other possibility we considered is that the discrepancy results from the different techniques used to detect tyrosine-phosphorylated proteins. In our previous work, 1-D gels were divided into a limited number of slices and the phosphoamino acid composition of each slice was determined. This technique does not adequately resolve between proteins with molecular masses of 42 and 45 kDa, and it is now known that tyrosine phosphorylation of a group of 44- to 45-kDa proteins is also stimulated by mitogen stimulation (13, 28, 28b). Since phosphorylation of these 44- to 45-kDa proteins has been best characterized by 2-D gel electrophoresis, we turned to this technique to determine whether TPA could stimulate tyrosine phosphorylation of at least one of these proteins in the TNR9 cells, a finding which would resolve the discrepancy with our earlier report.

TNR9 cells were labeled with $^{32}P_i$ and stimulated for 10 min with TPA. Cell proteins were separated by two-dimensional gel electrophoresis, transferred to Immobilon, and treated with KOH, to enrich for tyrosine phosphorylations (15, 25). TPA treatment of TNR9 cells induced the alkali-stable phosphorylation of a whole set of proteins in the 44- to 45-kDa area (Fig. 3B, arrows). Direct phosphoamino acid analysis of the spots cut from the alkali-treated filter re-

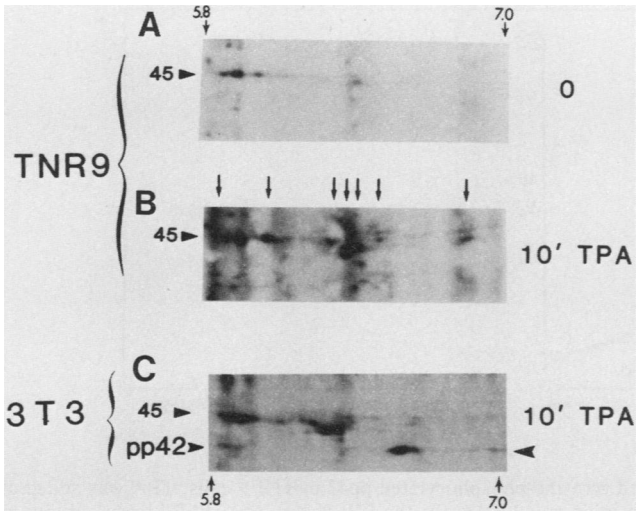

FIG. 3. 2-D gel analysis of ^{32}P-labeled proteins. (A) Control gel (no TPA). (B and C) TPA was added at 1 μg/ml for 10 min to 3T3 (C) and TNR9 (B) cells before lysis. Fifty micrograms of protein was loaded onto the first-dimension gels for each cell line. Overnight exposures of alkali-treated blots are shown and are representative of three experiments. Arrows indicate the phosphoproteins taken for phosphoamino acid analysis. Note that the incorporation of ^{32}P into the 44- to 45-kDa proteins is comparable to the incorporation of ^{32}P into pp42 in the 3T3 cells. By contrast, the immunoblotting procedure detects tyrosine-phosphorylated pp45 only weakly, compared with its ability to detect pp42 (Fig. 1). Apparently, the contexts of these tyrosine phosphorylations and/or the presence of other proteins at the same M_r dramatically affects the ability of the antisera to bind to the tyrosine-phosphorylated epitopes, as we have shown in other cases (28a). Because the recognition of specific tyrosine phosphorylations by antiphosphotyrosine antisera can be highly dependent on the specific phosphotyrosine-containing protein examined, it is important to recognize that the signals obtained in these immunoblots are not necessarily quantitatively proportional to the chemical amount of phosphotyrosine present in a protein. Indeed, we have no evidence that the tyrosine-phosphorylated 45-kDa protein recognized by the immunoblotting procedure shown in Fig. 1 corresponds to any of the ^{32}P-labeled 44- to 45-kDa proteins shown here. Numbers on the left of the gels are molecular masses (in kilodaltons).

vealed that over half the phosphoamino acid was present as phosphotyrosine (data not shown). By contrast, TPA treatment of 3T3 cells induced the alkali-stable phosphorylation not only of a series of 44- to 45-kDa proteins but also of pp42. We confirmed that the protein spot designated pp42 in Fig. 3 had the appropriate position by mixing it with purified MAP kinase, which (as shown previously [37]) migrated at the same M_r as the pp42 spot (data not shown). We conclude that the TPA-induced tyrosine phosphorylation of pp42 is defective in the 3T3-TNR9 cell variant but that tyrosine phosphorylation of at least some 45-kDa proteins occurs in these cells, a result which explains the apparent discrepancy with our earlier report (7). However, as discussed in the legend to Fig. 3, there is not a one-to-one correspondence between the alkali-stable ^{32}P-labeled proteins revealed in Fig. 3 and the proteins detected by blotting with antiphosphotyrosine antibodies (Fig. 1 and 2).

Phosphorylation and activation of MAP kinase. MAP kinase can be partially purified from EGF-stimulated 3T3 cells by anion exchange and hydrophobic-interaction chromatography and has been shown to copurify with the pp42 detected in whole-cell immunoblots (37). MAP kinase can similarly be purified from TPA-treated 3T3 cells and likewise copurifies with pp42 (Fig. 4). As we have previously reported for EGF activation (37), the only TPA-inducible tyrosine-phosphorylated protein found by immunoblotting the MAP kinase preparation with phosphotyrosine antibodies was the pp42 protein (Fig. 4B). The differential effect of TPA on MAP kinase phosphorylation in 3T3 versus TNR9 cells can be seen in this partially purified MAP kinase preparation. Applying the same purification and blotting procedure which reveals tyrosine-phosphorylated pp42 in the 3T3 cells, we failed to detect any tyrosine-phosphorylated MAP kinase in TNR9 cell homogenates even after a 20-fold-longer exposure (Fig. 4B). Thus, just as whole-cell lysates failed to reveal a rapid tyrosine phosphorylation of pp42 in the TPA-stimulated 3T3-TNR9 cells, so the purification procedure for MAP kinase failed to yield any 42-kDa tyrosine-phosphorylated protein from this source under the same conditions of activation.

We have considered the possibility that this poorly abundant protein (14) was differentially lost during purification from 3T3-TNR9 cells. However, even after loading twice as much TNR9 total lysate protein onto the FPLC phenyl Superose column and electrophoresing the entire fractions, we were unable to detect any tyrosine-phosphorylated pp42 protein in the TNR9 cell line stimulated by TPA for 10 min. (28b). We also considered the possibility that a variant form of pp42 might display a different elution profile on FPLC. However, we have also analyzed the tyrosine phosphorylation pattern of the 3T3-TNR9 fractions eluting before and after the elution position of the 3T3 MAP kinase without finding any pp42 detectable with the phosphotyrosine antibodies (not shown).

The absence of pp42 tyrosine phosphorylation in TPA-stimulated 3T3-TNR9 cells was not due to the absence of the protein itself in these cells, since the addition of PDGF to TNR9 cells led to the appearance of a tyrosine-phosphorylated protein of 42 kDa which possessed the same elution profile from phenyl Superose (Fig. 5) as the partially purified protein visualized in Fig. 4 from 3T3 cells or as the purified enzyme detected in 3T3-L1 cells (35, 36).

The defect in TPA-induced phosphorylation of pp42 in the 3T3-TNR9 cells is mirrored in defective enzymatic activation of MAP kinase (Fig. 4). Addition of TPA to Swiss 3T3 cells induced a large increase in phenyl Superose-purified MAP kinase activity as determined by phosphate incorporation into the microtubule-associated protein 2 substrate. By contrast, TPA treatment of TNR9 cells did not induce detectable MAP kinase activity either in the normal elution position of 3T3 MAP kinase or in any other fraction eluted through the phenyl Superose chromatography step. Even when we used a more sensitive assay method to assess this enzymatic activity (17), namely, the phosphorylation of myelin basic protein, no MAP kinase activity was detected in TPA-activated TNR9 cells (data not shown).

Since the MAP kinase showed the same elution profile from phenyl Superose whether the kinase had been isolated from EGF- or TPA-stimulated 3T3 cells (37; this study), from PDGF-stimulated 3T3-TNR9 cells (Fig. 5), or from insulin-stimulated 3T3-L1 adipocytes (35), and since pp42 is the only tyrosine-phosphorylated protein detectable in each of these fractions, we believe that the same protein is being studied in all these cases.

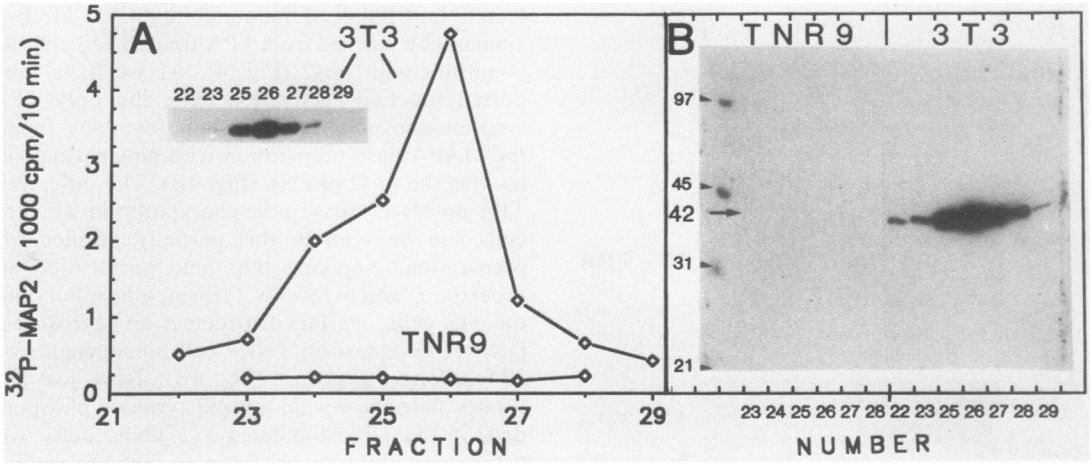

FIG. 4. Absence of TPA-stimulated MAP kinase activity and of purified tyrosine-phosphorylated pp42 in TNR9 cells. TPA was added at 300 ng/ml for 10 min to both 3T3 and TNR9 cells. A total of 0.5 mg of protein was loaded for the FPLC run. (A) MAP kinase activity in fractions from 3T3 and TNR9 cell lysates separated by phenyl-Superose FPLC was assayed with the microtubule-associated protein 2 substrate (^{32}P-MAP2). (Inset) Antiphosphotyrosine immunoblot of pp42 in the phenyl Superose fractions from 3T3 cells which displayed MAP kinase activity. Note the excellent correspondence between the peak of enzyme activity and the tyrosine-phosphorylated protein. (B) Autoradiogram of a phosphotyrosine immunoblot of the partially purified MAP kinase preparations from 3T3 and TNR9 cells shown in panel A, with a 20-fold-longer exposure. Numbers on the left are molecular masses (in kilodaltons).

DISCUSSION

Regulation and phosphorylation of MAP kinase/pp42. TNR9 cells were derived from Swiss 3T3 cells on the basis of their mitogenic nonresponsiveness to the tumor promoter TPA (11). Since these cells possess normal levels of functional PK-C, they presumably are defective in some aspect of PK-C-dependent intracellular signalling and thus provide a suitable system for studying this signalling system. We have found that tyrosine phosphorylation of pp42 and phosphorylation and activation of MAP kinase are both defective in TPA-treated TNR9 cells. These findings strengthen our earlier suggestion that MAP kinase is identical or closely related to pp42: not only do the two proteins copurify, as shown previously (37), but also their phosphorylation is coregulated. This is shown by the facts that the same agonists stimulate both pp42 phosphorylation and MAP kinase phosphorylation and activation and that in the TNR9 cells both are defective when TPA, but not PDGF, is the agonist.

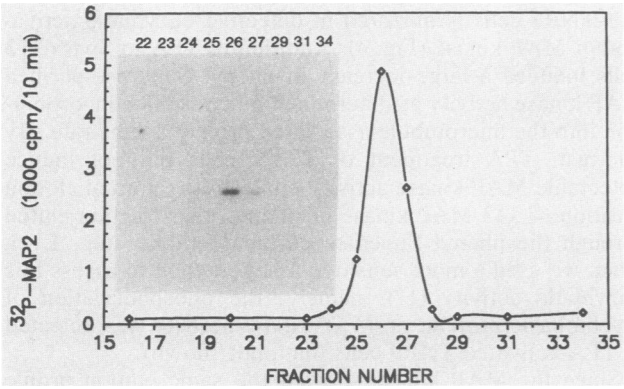

FIG. 5. Tyrosine phosphorylation and enzymatic activation of MAP kinase by PDGF in TNR9 cells. Samples from PDGF-treated TNR9 cells were processed and assayed as for Fig. 4. One milligram of protein was loaded onto the phenyl Superose column. MAP kinase activity is displayed graphically as microtubule-associated protein 2 phosphorylation by partially purified MAP kinase, and the antiphosphotyrosine immunoblot of this material is also shown (inset). PDGF was added at 10 ng/ml for 10 min.

The fact that MAP kinase can be activated by treatment of TNR9 cells with PDGF indicates that the protein is present in these variant cells. This finding has been confirmed by immunoblotting with an antibody raised against a protein which is related to MAP kinase and which was purified from sea star oocytes (38). These results indicate similar levels of the pp42 protein in 3T3 and TNR9 cells (28b, 36a).

Thus, the TNR9 cells possess MAP kinase but are defective in the PK-C-dependent portion of its regulation. This suggestion is based not only on the fact that TPA is unable to activate MAP kinase in these cells but also on the observation that down-modulation of PK-C blunts the ability of PDGF to induce pp42 phosphorylation in 3T3 but not in TNR9 cells. These results could be explained if there exists a family of closely related pp42 proteins responsive to differing signalling pathways and if the TNR9 cells lack one of these family members. However, we favor the hypothesis that TNR9 cells are defective at some step upstream of MAP kinase, perhaps in the regulation of a "MAP kinase kinase" protein.

TNR9 cells possess normal levels of PK-C, and the activity and translocation of this enzyme in response to TPA appear to occur normally (7, 18). A more recent report from Biemann and Erikson (5) indicates that the TPA-induced down-modulation of PK-C does not persist in TNR9 cells which are allowed to overgrow and form multilayers. However, neither the original TNR9 cells (11) nor the clones we work with overgrow and form multilayers, suggesting that the cells utilized in the studies of Biemann and Erikson may have undergone some additional changes. Thus, it is difficult to assess the relevance of their interesting result to the findings reported here.

Cellular functions of MAP kinase. The in vivo results described here support the in vitro findings which suggest

that MAP kinase is an important regulator of the activity of S6 kinase, since the TNR9 cells have previously been found to be defective in TPA-induced (but not serum-induced) activation of ribosomal S6 kinase (18). Thus, we suggest that MAP kinase is an enzyme which functions upstream of S6 kinases to control protein synthesis in response to diverse physiological signals involving either PK-C or tyrosine phosphorylation.

Tabarini et al. (40) and Blenis and Erikson (8) have demonstrated that TPA-stimulated S6 kinase possesses the same chromatographic properties as S6 kinase stimulated by peptide mitogens. However, the TPA-dependent activation pathway(s) for S6 kinase clearly differs from those triggered by serum factors since the down-modulation of PK-C by TPA did not completely block the stimulation of S6 kinase by serum (9). Since the down-modulation of PK-C has similar effects on the activation of MAP kinase, i.e., abolition of TPA response and partial preservation of EGF response (36b), it can be argued that MAP kinase has the diversity of control mechanisms required to explain the various S6 kinase responses without invoking the convergence of signalling pathways on S6 kinases themselves.

On the other hand, TPA treatment of TNR9 cells results in a normal induction of most of the measured changes in gene transcription, including *fos* and the TIS genes (TPA-induced sequences) (29, 30). Therefore, activation of MAP kinase appears not to be essential for these transcriptional changes.

Recently, Boulton et al. (10) reported the cloning and sequencing of a gene encoding a protein related to MAP kinase. This gene sequence has significant homology with the yeast kinases KSS1 and FUS3, two mediators of the pheromone-induced response in yeast. In addition, significant homology with the CDC28/cdc2 family of serine/threonine kinases exists. These results demonstrate the evolutionary conservation of a kinase family which plays a crucial role in controlling the entry into and exit from the cell cycle. The use of genetic variants such as the 3T3-TNR9 cells should help to further elucidate the regulation and function of this kinase family in animal cells.

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