Analysis of mitogen-activated protein kinase activation by naturally occurring splice variants of TrkC, the receptor for neurotrophin-3

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TrkC is a receptor tyrosine kinase that binds neurotrophin-3 (NT-3) with high affinity. A number of naturally occurring splice variants of TrkC exist, including one (TrkC.ki14) with a 14 amino acid insertion between subdomains VII and VIII of the tyrosine kinase domain. This kinase insert blocks the ability of NT-3 to stimulate neurite outgrowth in PC12 cells and proliferation in fibroblasts. The inserts also block the ability of TrkC to form a high-affinity complex with Shc and phospholipase Cγ (PLCγ) and the activation of PtdIns 3-kinase, and attenuates the sustained activation of mitogen-activated protein kinase (MAPK). In the current study we set out to determine whether the attenuation of the activation of MAPK by the insert was the result of the inability of TrkC to activate the Shc–Ras pathway, PtdIns 3-kinase activation, PLCγ activation, or a combination thereof. Experiments with the use of cell-permeant inhibitors argue against a major role for PLCγ and PtdIns 3-kinase in the activation of MAPK by TrkC. The introduction of the 14 amino acid kinase insert appeared to slow the kinetics of NT-3-stimulated Shc phosphorylation and Shc–Grb2 association and reduce their magnitude; an effect which was associated with a delayed, and only transient, activation of MAPK. Taken together, our data suggest that the apparent defect in MAPK activation caused by the kinase insert may result predominantly from an inhibition of high-affinity Shc binding, although a role for PLCγ and PtdIns 3-kinase cannot be completely excluded.

INTRODUCTION

Neurotrophin-3 (NT-3) plays a critical role in the development and survival of mammalian neurons. NT-3 is a member of a wider family of polypeptides collectively termed the neurotrophins, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT4/5) and neurotrophin-6 (NT-6). Whilst all neurotrophins bind a low-affinity transmembrane receptor, p75, the major signal-transducing components appear to be the transmembrane tyrosine kinases, i.e. TrkA (binds NGF), TrkB (binds BDNF, NT-3 and NT-4/5) and TrkC (binds NT-3) [1].

The binding of NT-3 to TrkC results in the autophosphorylation of five principle tyrosine residues. The role of these tyrosines in signalling has been extensively reviewed [1]. Three of the tyrosines (674, 678, 679) probably play a role in activation of the kinase towards exogenous substrates. Phosphorylation of tyrosine 485 allows the phosphotyrosine-binding domain of Shc to interact with TrkC, resulting in tyrosine phosphorylation of Shc and its consequent interaction with the Grb2–Sos complex. This association then stimulates the activation of the Ras → Raf-1 → mitogen-activated protein kinase (MAPK) kinase → MAPK cascade. The phosphorylation of tyrosine 789 results in the additional binding of phospholipase Cγ (PLCγ) via its SH2 domain. PLCγ is then phosphorylated and activated. PtdIns 3-kinase is also activated, although the mechanism by which this occurs is obscure.

Little is known about the role of PLCγ in neurotrophic factor signalling. Neurotrophic factors promote an increase in Ins(1,4,5)P_3, and there is evidence for changes in cytosolic Ca^{2+} ion concentrations and the activation of protein kinase C (PKC) [2–4]. It is well established that phorbol esters acting through PKC can activate the MAPK cascade, probably through the phosphorylation and activation of Raf-1 [5]. In some cell types, wortmannin, an inhibitor of PtdIns 3-kinase, has been shown to inhibit MAPK activation by receptor tyrosine kinases [6,7]. In conclusion, there may be at least three pathways by which NT-3 could activate the MAPK cascade: one via Shc → Grb2 → Sos → Ras → Raf-1, another via PLCγ → diacylglycerol → PKC → Raf-1 and a further poorly characterized pathway involving PtdIns 3-kinase.

The rat TrkC locus encodes a number of differentially spliced isoforms which lack the cytoplasmic tyrosine kinase domain and others that exist as splice variants. These variants possess inserts of variable length, with 14 (TrkC.ki14), 25 (TrkC.ki25) and 39 amino acid inserts (TrkC.ki39) occurring between subdomains VII and VIII of the tyrosine kinase domain [8–10]. These inserts block the ability of TrkC to mediate NT-3-stimulated neurite outgrowth in PC12 cells and proliferation in fibroblasts [8–11].

In fibroblasts over-expressing TrkC, NT-3 promotes sustained activation of MAPK, which is prevented upon introduction of the kinase inserts; for example TrkC.ki14 only promotes a transient activation of MAPK [11]. In PC12 cells it has been reported that NGF-induced neurite outgrowth requires a sustained activation of MAPK [12,13]. The fact that the kinase insert variants promote only a transient MAPK activation could explain their inability to mediate NT-3-dependent neurite outgrowth in PC12 cells.

The lack of sustained MAPK activation by TrkC.ki14 in fibroblasts is associated with a lack of autophosphorylation of tyrosine 789, and thus reduced PLCγ association and tyrosine phosphorylation [11]. Tyrosine phosphorylation of Shc was apparently unaffected by the kinase inserts; however, Shc no longer formed a stable complex with TrkC [11]. Finally, PtdIns 3-kinase activation was blocked by the kinase inserts [9]. These observations suggest that the lack of Shc binding, PLCγ binding and activation, and PtdIns 3-kinase activation, might contribute to the ability of the kinase inserts to attenuate sustained MAPK

Abbreviations used: BDNF, brain-derived neurotrophic factor; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PLCγ, phospholipase Cγ; NT-3, neurotrophin-3; GST, glutathione S-transferase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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activation in fibroblasts [11]. As our previous data could not distinguish between these three possibilities, we set out in this study to perform a more detailed analysis of the role of Shc, PtdIns 3-kinase and PLCγ in the activation of MAPK by TrkC and TrkC.ki14 in fibroblasts.

MATERIALS AND METHODS

Materials

Polyclonal anti-Trk C-terminal peptide antibody (immunoreactive toward TrkA, TrkB and TrkC) was as described [14]. Antisera towards phosphotyrosine (4G10) and Grb2 were obtained from Upstate Biotechnology Inc. (UBI, Lake Placid, NY, U.S.A.). Anti-Shc SH2 domain antibodies were kindly supplied by Professor K. Siddle (University of Cambridge, Cambridge, U.K.). Anti-p70S6 kinase antibodies were provided by E. Foulstone (University of Bristol, Bristol, U.K.). Immobilion-P was from Millipore Corp. Unless otherwise stated all biochemicals were from Sigma (Poole, Dorset, U.K.), and general purpose laboratory reagents were of analytical grade and were obtained from BDH (Poole, Dorset, U.K.). MG87 cells (an NIH 3T3 subclone) expressing TrkC or TrkC.ki14 were maintained and cultured as previously described [11].

Extraction of cells and Western blot analysis

Cells were grown to approx. 80% confluence in 60-mm dishes using Dulbecco's modified Eagle's medium with 10% (v/v) calf serum and 500 μg/ml G418. The cells were then serum-starved for 2 h before incubation with 50 ng/ml NT-3 for the times indicated in the Figure legends. Cell lyates were prepared and proteins were immunoprecipitated as previously described [15], using the antibodies stated in the Figure legends. The immunoprecipitates were run on SDS/7%-PAGE gels, transferred electrophoretically to Immobilion-P, and Western blotting was performed as described previously [14], but using the BM Chemiluminescence system (Boehringer Mannheim) and with the antibodies described in the Figure legends. Densitometric analysis of the blots was performed using a Joyce Loebel Densitometric Scanner.

MAPK assays

Transfected fibroblasts were grown to approx. 80% confluence in 60-mm dishes. Cells were serum-starved for 2 h before incubation with 50 ng/ml NT-3 for the times indicated in the Figure legends. Cells were washed and extracted, and MAPK activity in the cell lysate was assayed in the presence of [γ-^{32}P]ATP, as described by Young et al. [16] but using 200 μM T669 peptide as substrate. This peptide is based on the sequence surrounding the MAPK phosphorylation site in the epidermal growth factor receptor (KRELVEPLTPSGEAPNQALLR).

MAPK kinase assays

The samples prepared for MAPK assays were assayed for MAPK kinase activity in parallel. Assays were set up essentially as for the MAPK assays but the T669 peptide was substituted with 1 μg of purified recombinant glutathione S-transferase (GST)–MAPK. Each assay was carried out for 30 min at 30 °C. The reactions were terminated by the addition of SDS/PAGE sample buffer, boiled for 3 min and then separated on an SDS/7%-PAGE. The gel was stained, destained and dried, and the incorporation of ^{32}P into the GST–MAPK substrate was detected and quantified by exposure using a Molecular Dynamics PhosphoImager.

p70S6 kinase assays

Cell extracts used for assaying MAPK were also assayed for p70S6 kinase. p70S6 kinase was immunoprecipitated using an antibody specific to p70S6 kinase, and activity of this kinase was assayed as described by Moule et al. [17] using the peptide KEAKEKRQEQIAKKRRLSSLRASTSKSESSQK, which is based on the sites phosphorylated by this enzyme in the ribosomal S 6 protein.

RESULTS

Time course of receptor phosphorylation

The fibroblast cell line MG87, which lacks endogenous Trk family members, expresses equivalent levels of TrkC or TrkC.ki14 [11]. We first assessed the kinetics of receptor tyrosine phosphorylation after stimulation with NT-3 over a 2 h time period. Western blot analysis demonstrated that the initial phase of TrkC tyrosine phosphorylation was faster than that of TrkC.ki14 and that the maximal level of phosphorylation of TrkC attained was greater than for TrkC.ki14 (Figure 1 and Figure 4A). A period of TrkC dephosphorylation then occurs. Indeed, between 10 and 120 min the tyrosine phosphorylation states of TrkC and TrkC.ki14 were virtually indistinguishable. We have previously reported, at least at the 5 min time point, that the reduced level of tyrosine phosphorylation of TrkC.ki14 compared with TrkC is due solely to the reduction in phosphorylation of tyrosine 789, the PLCγ interacting site.

Time course of Shc phosphorylation

The binding and tyrosine phosphorylation of Shc is one of the earliest events to occur after the autophosphorylation of the Trk family [1,18]. NT-3-induced Shc tyrosine phosphorylation was determined over an identical time period to that used above. We found that all three isoforms of Shc were phosphorylated in response to NT-3 in these fibroblasts (Figure 2 and Figure 4B). This analysis demonstrated that the initial rate of tyrosine phosphorylation of Shc in response to NT-3 in cells expressing TrkC.ki14 was delayed and reached a lower maximal level when compared with cells expressing TrkC (Figure 2 and Figure 4B). As with receptor tyrosine phosphorylation, between 20 and 120 min the tyrosine phosphorylation of Shc induced by NT-3 was virtually identical in cells expressing TrkC and TrkC.ki14.

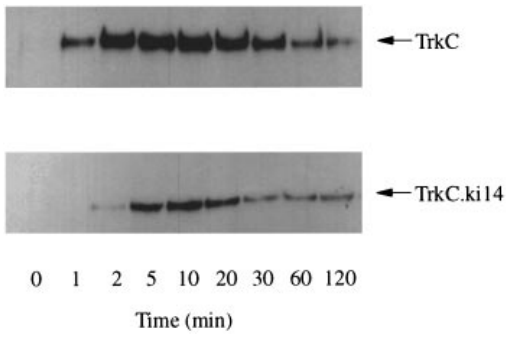

Figure 1 Time course of TrkC and TrkC.ki14 phosphorylation

MG87 cells expressing full-length TrkC or TrkC.ki14 were incubated in the presence of NT-3 (50 ng/ml) and extracted at the times indicated. Receptors were immunoprecipitated with specific antisera. The precipitates were separated by SDS/PAGE and Western blotted with anti-phosphotyrosine antisera, followed by detection by enhanced chemiluminescence (ECL).

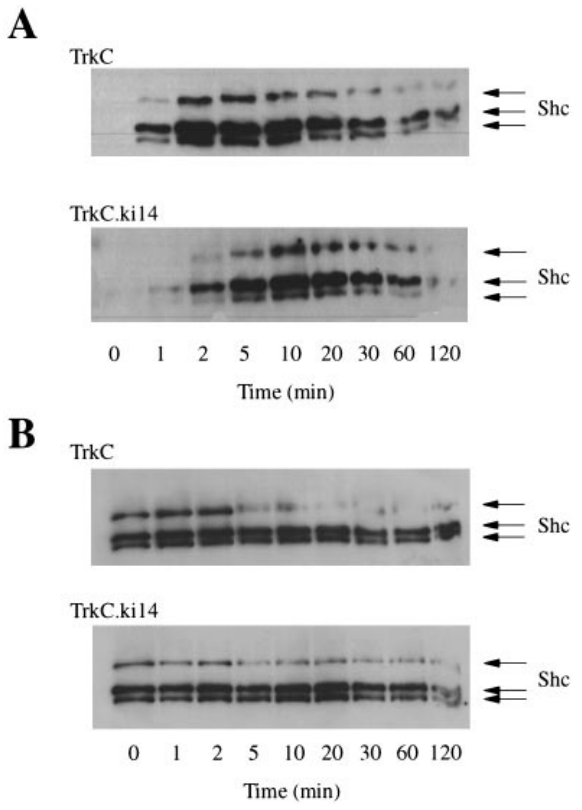

Figure 2 Time course of Shc phosphorylation after stimulation via TrkC and TrkC.ki14

MG87 cells expressing full-length TrkC or TrkC.ki14 were incubated in the presence of NT-3 (50 ng/ml) and extracted after the times indicated. Shc proteins were immunoprecipitated with specific antisera. The precipitates were divided in two and separated by SDS/PAGE and Western blotted with anti-phosphotyrosine antisera (**A**) or anti-Shc antisera (**B**), followed by detection by ECL.

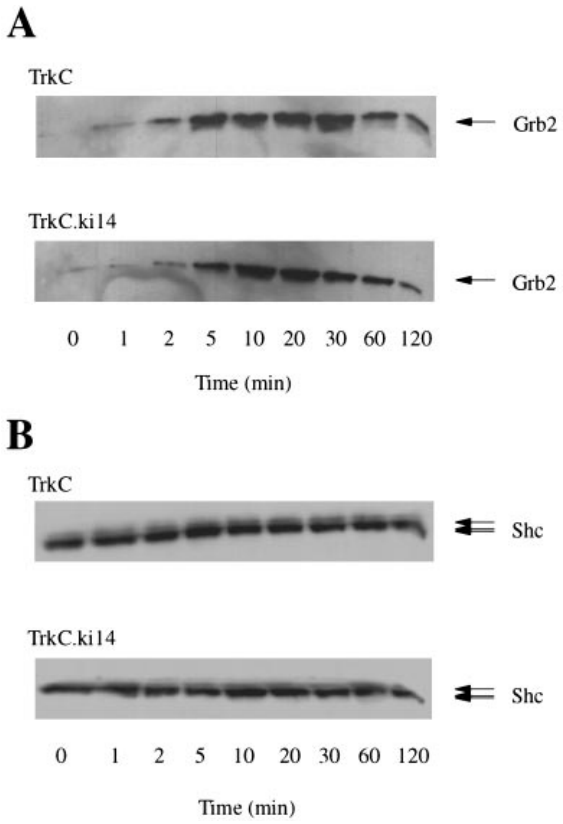

Figure 3 Time course of Grb2 association with Shc after NT-3 stimulation via TrkC and TrkC.ki14

MG87 cells expressing full-length TrkC or TrkC.ki14 were incubated in the presence of NT-3 (50 ng/ml) and extracted at the times indicated. Shc proteins were immunoprecipitated with specific antisera. The precipitates were separated by SDS/PAGE and Western blotted with anti-Grb2 antisera, followed by detection by ECL (**A**). The membranes were then re-probed with anti-Shc sera, followed by ECL detection (**B**).

Time course of Grb2 association with phosphorylated Shc

Shc, once it is tyrosine phosphorylated, associates with the Grb2 adaptor protein via the SH2 binding motif of the latter [18]. Anti-Shc immunoprecipitates were probed for the presence of Grb2 (Figure 3 and Figure 4C). The kinetics of Shc association with Grb2 exhibited a similar trend to that of both receptor and Shc tyrosine phosphorylation, i.e. delayed initial rate and lower peak phosphorylation were attained in cells expressing TrkC.ki14. Again, a period of Shc–Grb2 dissociation then occurred, and the extent of this association between 60 and 120 min were almost identical in cells expressing TrkC and TrkC.ki14. Interestingly, however, the association of Grb2 with Shc was slightly delayed compared with the tyrosine phosphorylation of Shc in cells expressing TrkC or TrkC.ki14 (see Figure 3 and Figure 4C). This delay may be explained by a translocation of Grb2 to tyrosine phosphorylated Shc, a process that requires 1–2 min to occur.

Time course of MAPK kinase activation

Having measured some of the most immediate signalling responses from these two different receptors, we went on to measure signalling events further downstream in the Raf-1 → MAPK → kinase MAPK cascade. A time course of MAPK kinase activation again showed a significant difference between cells expressing TrkC or TrkC.ki14 (Figure 5). Activation of TrkC, resulted in a large and rapid response, peaking at 5 min. This initial phase was followed by a decline in activity by 20 min, which was then followed by a second slower phase of activation over the next 100 min (Figure 5). This second phase of MAPK kinase activation continued for at least a further 2 h (results not shown). In contrast, TrkC.ki14 mediated only a very small and transient activation of MAPK kinase in response to NT-3; no evidence for a second phase of activation was evident.

Time course of MAPK activation

TrkC mediated two phases of MAPK activation parallel to that seen with MAPK kinase (see also [11]). In contrast, TrkC.ki14 mediated only the initial phase, albeit reaching a lower maximal level than that observed with TrkC (Figure 5). Again, as with MAPK kinase, no evidence for the second slower phase of MAPK activation was observed for cells expressing TrkC.ki14, although activity was still significantly above basal after 2 h.

Effect of RO-31-8220 and wortmannin on MAPK activition by NT-3

The activation of PLCγ results in Ins(4,5)P_2 hydrolysis, yielding Ins(1,4,5)P_3 and diacylglycerol. The latter could result in the activation of MAPK via a PKC Raf-1-dependent mechanism. While MAPK can also be activated via Ca^{2+}-dependent mechanisms this possibility is excluded in our cells, as we observe

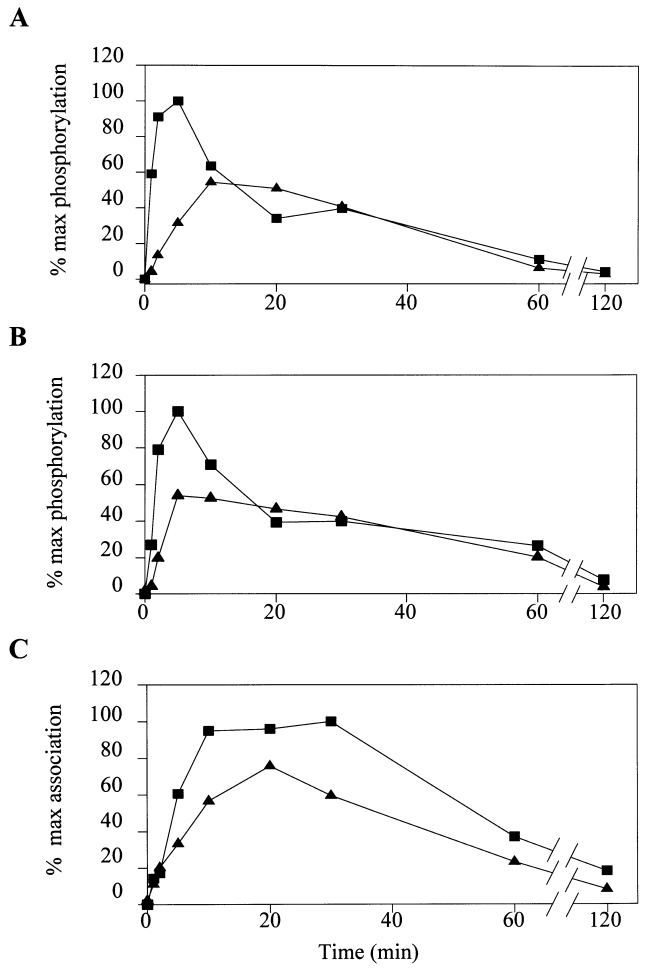

Figure 4 Comparison of receptor and Shc phosphorylation and Grb2 association to Shc, after NT-3 stimulation via TrkC and TrkC.ki14

The Western blots from Figures 1, 2 and 3 were quantified by densitometry, and the level of Shc phosphorylation (**B**) and Grb2 association with Shc (**C**) is expressed after correcting for the amount of Shc immunoprecipitated. In (**A**), quantification of the amount of TrkC immunoprecipitated was not possible due to contamination of precipitates with IgG. All data are expressed as a percentage of the maximal response seen in the two cell lines: ■, stimulation via TrkC; ▲, stimulation via TrkC.ki14.

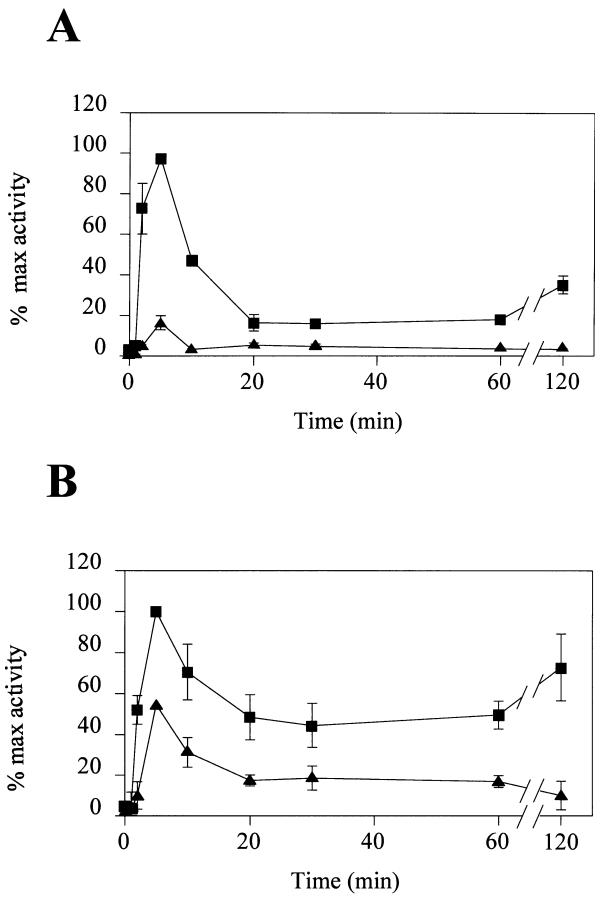

Figure 5 Activation of MAPK kinase and MAPK by NT-3 in cells expressing either TrkC or TrkC.ki14

MG87 cells expressing either TrkC or TrkC.ki14 were incubated with NT-3 (50 ng/ml) for the times indicated. The cells were lysed and the activity of MAPK kinase assayed in the resulting extract using the recombinant substrate GST–MAPK (**A**). With the same samples, the activity of MAPK was measured using the EGFR669 peptide (**B**). The results are representative of three separate experiments and expressed as a percentage of the maximum activity attained ± S.E.M.: ■, stimulation via TrkC; ▲, stimulation via TrkC.ki14.

Table 1 Effects of PKC inhibitor, RO-31-8220, on MAPK activity

MG87-TrkC cells were incubated for 20 min in the presence or absence of 100 nM RO-31-8220 before the addition of either NT-3 (50 ng/ml) or PMA (1.6 μM). The cells were extracted at the times indicated and MAPK activity was measured as previously described. The results (mean ± S.E.M.) of three separate experiments are shown, each performed in duplicate.

	Percentage of maximal response			
	NT-3		PMA	
RO-31-8220 addition ...	−	+	−	+
Stimulation time (min)				
5	100	94.2 ± 11.4	82.7 ± 3.8	2.9 ± 1.9
120	31.8 ± 7.8	53.7 ± 14.8	44.9 ± 3.2	9.1 ± 3.9

no changes in intracellular Ca^{2+} ion concentrations using fura 2 measurements [11]. To explore a possible role for PKC, and thus by inference PLCγ, in the activation of MAPK by NT-3 we used RO-31-8220, a relatively specific inhibitor of PKC. RO-31-8220 (at 100 nM) almost completely blocked the ability of phorbol 12-myristate 13-acetate (PMA) to activate MAPK in cells expressing TrkC (Table 1). On the other hand this inhibitor had no effect on MAPK activation by NT-3, during either the early or late phase.

Effect of wortmannin on the activation of MAPK and p70S6 kinase by NT-3

It has been reported that PtdIns 3-kinase may play a role in the activation of MAPKs by some growth factors [6,7], and also that the TrkC kinase inserts block the activation of PtdIns 3-kinase by NT-3 [8]. We investigated whether PtdIns 3-kinase plays any role in the activation of MAPK by NT-3 in cells expressing TrkC. As shown in Table 2, wortmannin had no apparent effect on NT-3-stimulation of MAPK activity during either the early or late phases. Under these conditions we confirmed that wortmannin blocked the activation of p70S6 kinase by approx. 75% (Table 2).

Table 2 Effects of PtdIns 3-kinase inhibitor, wortmannin, on MAPK activity

MG87-TrkC cells were incubated for 20 min in the presence or absence of 100 nM wortmannin before the addition of NT-3 (50 ng/ml). The cells were extracted at the times indicated and divided in two. One fifth of the extract was used for measuring MAPK activity, as described in the Materials and methods section. Using a specific antibody, p70S6 kinase was immunoprecipitated from the remaining sample and its activity assayed using a peptide substrate (see the Materials and methods section). The results shown are of two separate experiments (mean ± S.D.) each performed in duplicate.

	Percentage of maximal response			
	MAPK assay		p70S6 kinase assay	
Wortmannin addition ...	−	+	−	+
Stimulation time (min)				
0	0.0	0.0	16.0 ± 0.3	27.2 ± 0.4
5	100.0	101.3 ± 10.8	100.0	26.5 ± 5.5
120	81.3 ± 7.2	64.2 ± 8.1	150 ± 41	29.9 ± 5.9

DISCUSSION

In our previous work we found that the ability of TrkC to mediate NT-3-stimulated proliferation and c-*fos* and c-*myc* gene transcription were blocked by the naturally occurring 14 amino acid insert within the tyrosine kinase domain [11]. In addition, TrkC mediated a sustained activation of the Erk isoforms of MAPK while the TrkC.ki14 variant only promoted a transient activation [11]. In the current study we found a more profound difference in signalling between TrkC and TrkC.ki14 when we measured MAPK kinase activation (Figure 5A). NT-3 stimulation of TrkC resulted in an activation profile of MAPK kinase which very closely paralleled that of MAPK activity (Figure 5B); both exhibited two phases of activation, i.e. an initial rapid phase, which peaked at 5 min, followed by a slower phase of activation over the next 2 h (Figure 5). These sustained activation profiles are reminiscent of those seen in PC12 cells treated with NGF [12] or cAMP analogues [16], both of which lead to neurite outgrowth. Indeed, the second sustained phase of activation is also seen in rat cerebellar granule cells treated with BDNF, which binds to the related receptor tyrosine kinase TrkB (F. J. Gunn-Moore, unpublished work).

This was in stark contrast to the profile of MAPK activation elicited by TrkC.ki14: here NT-3 promoted only a small and transient activition of MAPK kinase. This was accompanied by a pronounced initial phase of MAPK activation, although peaking at lower levels than seen in cells expressing TrkC, but no second and slower sustained phase of activation was observed. These data provide additional evidence for amplification in the MAPK cascade (i.e. a small activation of MAPK kinase can lead to substantial activation of MAPK). Sustained MAPK activation has been reported to be responsible for the proliferative response of CCL39 cells [19], thus the ability of the kinase insert to block the second sustained phase of MAPK activation by NT-3 may be responsible for the inability of this variant to promote cell growth in MG87 fibroblasts [11].

The inability of TrkC.ki14 to fully activate these downstream signalling events in fibroblasts was accompanied by an almost complete lack of phosphorylation of PLCγ, its activation [11] and PtdIns 3-kinase activation [8]. Theoretically, at least, MAPK could also be activated by PLCγ- and/or PtdIns 3-kinase-dependent mechanisms (see the Introduction). However our current data, combined with those of others, argue against a major role for PLCγ and PtdIns 3-kinase in MAPK activation by TrkC in fibroblasts. First of all, we found that the PKC inhibitor, RO-31-8220, abolished the stimulation of MAPK by PMA but did not alter either the early or late phases of MAPK activition by NT-3 (Table 1); note also that we have also found no significant changes in cytosolic Ca^{2+} ion concentrations despite a 4-fold increase in Ins(1,4,5)P_3 production ([11]; M. Guiton, F. J. Gunn-Moore and J. M. Tavaré, unpublished work). Secondly, we found wortmannin to have no effect on MAPK activation by NT-3 in cells expressing TrkC (Table 2). On the basis of this we propose that Shc binding and phosphorylation is sufficient for sustained MAPK activation in the context of TrkC. Our data are consistent with those of Baxter et al. [20]. They found that the sustained activation of MAPK by a platelet-derived growth factor receptor ectodomain and a TrkA tyrosine kinase domain chimera, was unaffected by a Y785F mutation in the kinase domain that blocked PLCγ association and phosphorylation.

Our data also suggest that the attenuation of MAPK activation by the kinase insert may also be primarily the result of the decrease in high-affinity Shc binding (see [11]). Again, Baxter et al. found that a Y490F mutation at the Shc binding site had a remarkably similar effect on the kinetics of MAPK activation [20], as observed with the kinase insert (Figure 5B). However, it should be stressed that we cannot completely exclude the possibility that defects in PLCγ and PtdIns 3-kinase activation do result, at least in part, in the defect in MAPK activation caused by the kinase insert. In order to exclude these possiblities we would have to restore normal PLCγ phosphorylation/activation and/or PtdIns 3-kinase activation in the context of TrkC.ki14.

Other aspects of Shc phosphorylation are of particular interest. We have previously reported that Shc phosphorylation by TrkC.ki14 (measured at 5 min post-NT-3 addition) was near normal but was incapable of forming a high-affinity complex with the receptor [11]. A more detailed examination of Shc tyrosine phosphorylation (Figure 2 and Figure 4B) revealed that the initial rate of Shc tyrosine phosphorylation was inhibited by the kinase insert, as was the maximal level of tyrosine phosphorylation attained. Despite this, the kinetics of dephosphorylation of Shc were unaffected by the kinase insert. In fact, the phosphorylation/dephosphorylation kinetics of Shc were almost exactly mirrored by tyrosine phosphorylation of TrkC and TrkC.ki14 themselves (compare Figures 1 and 2, and Figures 4A and 4B). Also, rather similar kinetics of association/dissociation between Shc and Grb2 were observed (Figure 3 and Figure 4C). However, the association of Grb2 with Shc always lagged behind that of Shc tyrosine phosphorylation, suggesting that the rate-limiting step in the formation of this complex was not Shc tyrosine phosphorylation but, perhaps, a translocation of Grb2 to tyrosine phosphorylated Shc; this would be expected to be within the locality of TrkC itself, i.e. the plasma membrane. Shc phosphorylation, Shc–Grb2 association, and MAPK activation all exhibited a slower activation and lower maximum in cells expressing TrkC.ki14. This strongly suggests that Shc is the major determinant of MAPK activation in response to neurotrophic factor stimulation.

While there is ample evidence in the literature to provide a link between Shc tyrosine phosphorylation and MAPK kinase activation (see the Introduction for references), our data suggest that there are two discrepancies which require explanation. Firstly, how does a relatively robust, albeit delayed, Shc tyrosine phosphorylation and Shc–Grb2 association, lead to only a very weak activation of MAPK kinase? The most likely explanation derives from the inability of Shc to form a high-affinity complex with TrkC.ki14 [11]. This would prevent relocation of Shc, and by inference Grb2 and Sos, from their location in unstimulated

cells, to RasGDP in NT-3-stimulated cells. Secondly, what causes the second phase of MAPK kinase and MAPK activation by NT-3 in cells expressing TrkC? During this time period (beyond 20 min) a decrease in TrkC and Shc tyrosine phosphorylation, and Shc–Grb2 association are observed; i.e. no such second phase occurs at steps close to the receptor. Our data do not provide an explanation for this phenomenon. The slow rate of appearance of the second phase suggests, perhaps, an increase in the expression level of MAPK kinase or a decrease in expression of a phosphatase specific for MAPK kinase, MAPK or Raf-1, perhaps as a result of a decrease in their rate(s) of gene transcription.

In conclusion, our results suggest that the activation of MAPK by TrkC is primarily dependent on the ability of Shc to form a high-affinity complex with tyrosine 485. Based on inhibitor studies in cells expressing TrkC, PLCγ and PtdIns 3-kinase play little detectable role in MAPK activation. Studies with cells expressing TrkC.ki14, suggest that the 14 amino acid kinase insert causes a decrease in the rate of Shc phosphorylation and its association with Grb2, which in turn leads to an attenuation of the activation of MAPK kinase and MAPK. In the context of reduced Shc association with TrkC.ki14, an additional role for PLCγ and/or PtdIns 3-kinase in the attenuation of MAPK activation cannot be excluded.

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