Serine phosphorylation and maximal activation of STAT3 during CNTF signaling is mediated by the rapamycin target mTOR

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Neuropoietic cytokines such as ciliary neurotrophic factor (CNTF) can activate multiple signaling pathways in parallel, including those involving Janus kinase (JAK)–signal transducers and activators of transcription (STATs) [1], mitogen-activated protein kinase (MAPK) [2], phosphatidylinositol 3-kinase (PI 3-kinase) and mammalian target of rapamycin (mTOR)–p70 S6 kinase [3]. Crosstalk occurs between these pathways, because studies have shown that STAT3 requires phosphorylation on tyrosine and serine residues by independent protein kinase activities for maximal activation of target gene transcription [4]. Members of the JAK/Tyk family of tyrosine kinases mediate phosphorylation of STAT3 at Tyr705 during CNTF signaling; however, the kinase responsible for phosphorylation at STAT3 Tyr727 appears to depend on both the extracellular stimulus and the cellular context [5–8]. Here we investigate the kinase activity responsible for phosphorylation of STAT3 on Ser727 in CNTF-stimulated neuroblastoma cells. We found that CNTF-induced phosphorylation of Ser727 was inhibited by the mTOR inhibitor rapamycin, but not by Ser727 in a CNTF-dependent manner by mTOR, but not by a kinase-inactive mTOR mutant or by p70 S6 kinase. In agreement with these biochemical studies, rapamycin treatment of cells transfected with a STAT-responsive promoter reporter decreased activation of the reporter to the same degree as a STAT3 Ser727Ala mutant. The ability of mTOR to contribute to activation of STAT3 extends the function of mTOR [9] in mammalian cells to include transcriptional regulation.

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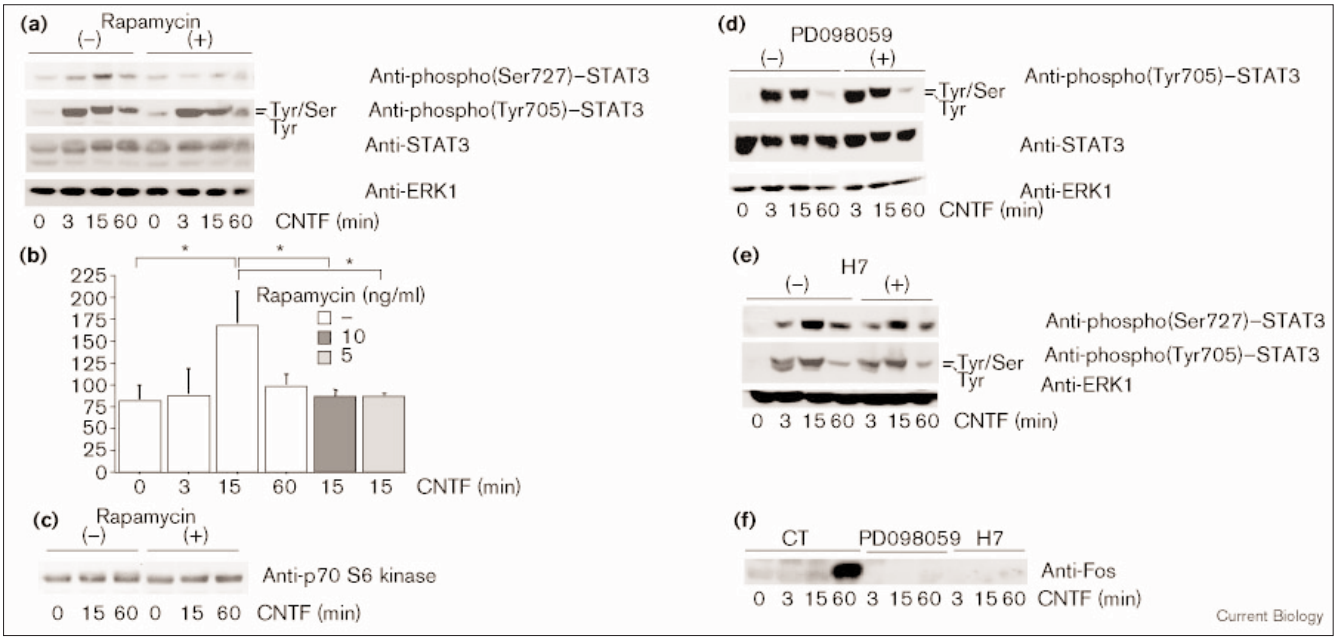
Results and discussion

The CNTF-related cytokine LIF has been shown to activate the mTOR/p70 S6 kinase/4E-BP1 pathway in cardiac myocytes [3]. We therefore examined whether this pathway mediated Ser727 phosphorylation of STAT3 during CNTF stimulation of human neuroblastoma NBFL cells. Rapamycin has been shown to inhibit the mTOR serine/threonine kinase, and the downstream mTOR effectors p70 S6 kinase and 4E-BP1 [10]. Rapamycin treatment of NBFL cells suppressed CNTF-induced phosphorylation of STAT3 on Ser727 to background levels as measured by immunoblotting analysis using an antibody to phospho(Ser727)-STAT3 and by the lack of the mobility shift characteristic of STAT3 serine phosphorylation (Figure 1a). Rapamycin treatment (5 ng/ml and 10 ng/ml) also abolished CNTF-induced p70 S6 kinase activity (Figure 1b) as well as the appearance of the phosphorylated form of p70 S6 kinase (Figure 1c). We and others have previously shown that CNTF stimulation of NBFL cells results in MAPK activation, leading to c-*fos* induction [2,11]. Treatment of CNTF-stimulated NBFL cells with

STAT3 when compared with cells stimulated with CNTF alone (Figure 1d,e). Downregulation of PKC by phorbol 12-myristate 13-acetate pretreatment also did not alter the migration of STAT3 (data not shown). We confirmed that the concentration of PD098059 and H7 used in these experiments was sufficient to inhibit activation of MAPK and a H7-sensitive kinase such as PKC by probing a filter with anti-Fos antibody. CNTF-stimulated induction of c-*fos* was completely blocked by 10 μM PD098059 and 100 μM H7 (Figure 1f). The MAPK family members SAPK/JNK and p38 were not detectably activated in CNTF-stimulated NBFL cells (data not shown). Thus the CNTF-activated kinase that mediates Ser727 phosphorylation of STAT3 in NBFL cells is either mTOR or a downstream kinase that is activated by mTOR, such as p70 S6 kinase.

To examine whether mTOR or p70 S6 kinase can mediate phosphorylation of STAT3 at Ser727 we immunoprecipitated these kinases from CNTF-stimulated cells and subjected the immunoprecipitates to *in vitro* kinase assays using a STAT3 peptide corresponding to residues 720–731 (STAT3(720–731)). The purified fusion protein GST–4E-BP1 and a S6 peptide were included as positive controls for mTOR and p70 S6 kinase activity, respectively. A peptide corresponding to the PKC recognition motif was used as a negative control. We used human embryonic kidney cell

Figure 1

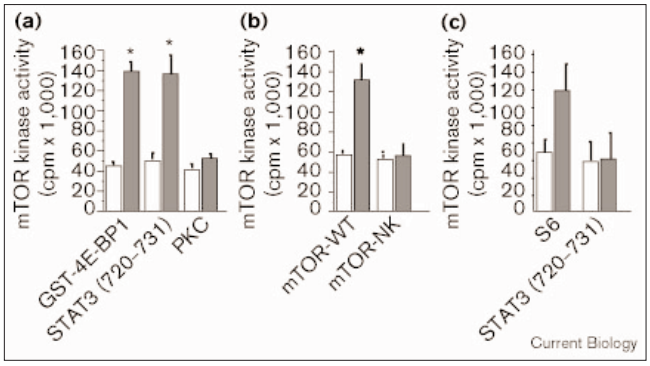


Activation of the mTOR/p70 S6 kinase/4E-BP1 pathway in CNTF stimulated NBFL cells and rapamycin-sensitive phosphorylation of STAT3 at Ser727. **(a)** Total cell lysates from cells not treated (–) or pretreated (+) with rapamycin (5 ng/ml) for 60 min and then stimulated with CNTF for the indicated times were immunoblotted and probed with the indicated antibody. The membrane was stripped and reprobed with anti-ERK1 antibody to verify equal amounts of protein were loaded on the gel. **(b)** CNTF-induced p70 S6 kinase activity was assessed with or without rapamycin pretreatment. Each time point was performed in triplicate. Data are expressed as mean ± SD. *$p < 0.05$ with reference to the indicated comparisons. **(c)** Total cell lysates from (a) were immunoblotted and probed with anti-p70 S6 kinase antibody. **(d,e)** PD098059 (d) or H7 (e) do not inhibit serine phosphorylation of STAT3 in NBFL cells. NBFL cells were either not treated (–) or pretreated (+) with PD098059 (10 μM) or H7 (100 μM) for 60 min. Cells were then stimulated with CNTF for the indicated times. Equal amounts of lysate were subjected to immunoblotting analysis and probed with indicated antibodies. The membranes were then stripped and reprobed with anti-STAT3 or anti-ERK1 antibody to verify that equal amounts of protein were loaded on the gel. **(f)** Cells were pretreated with PD098059 or H7 and processed as in (d,e) and lysates were immunoblotted and probed with anti-Fos antibody. CT, empty expression vector as control.

line HEK293T in these experiments because these cells respond to CNTF stimulation by activating JAK/STAT and mTOR/p70 S6 kinase/4E-BP1 pathways, but not the Ras/MAPK pathway. This is shown in the supplementary figure where CNTF stimulation of HEK293T cells results in Tyr705 and Ser727 phosphorylation of STAT3 (see Supplementary material; Figure S1b), and Ser/Thr phosphorylation of p70 S6 kinase, which is inhibited by pretreatment of the cells with rapamycin (Figure S1b). In contrast, CNTF does not induce phosphorylation of MAPK or increased levels of c-Fos (Figure S1c). HEK293T cells were transfected with an expression vector encoding hemagglutinin (HA)-tagged wild-type mTOR (mTOR-WT) or kinase-inactive mTOR (mTOR-NK) [12], stimulated with CNTF, and then immunoprecipitated with anti-HA antibody. Immunoprecipitated mTOR-WT but not mTOR-NK phosphorylated the STAT3(720–731) peptide and GST–4E-BP1 protein to the same degree (Figure 2a,b). There was no significant increase in phosphorylation of the PKC substrate peptide over that in unstimulated cells. In addition, although endogenous p70 S6 kinase immunoprecipitated from CNTF-stimulated cells efficiently phosphorylated the control S6 peptide, it did not phosphorylate STAT3(720–731) to a greater degree than observed in unstimulated cells (Figure 2c). The consensus recognition motifs have been determined for mTOR (Ser/Thr)-Pro [13] and p70 S6 kinase (X–X–Arg/Lys–X–Arg–X–X–Ser–X–X; where X is any amino acid) [14] and the motif recognized by mTOR is in agreement with the Ser727–Pro728 region of STAT3.

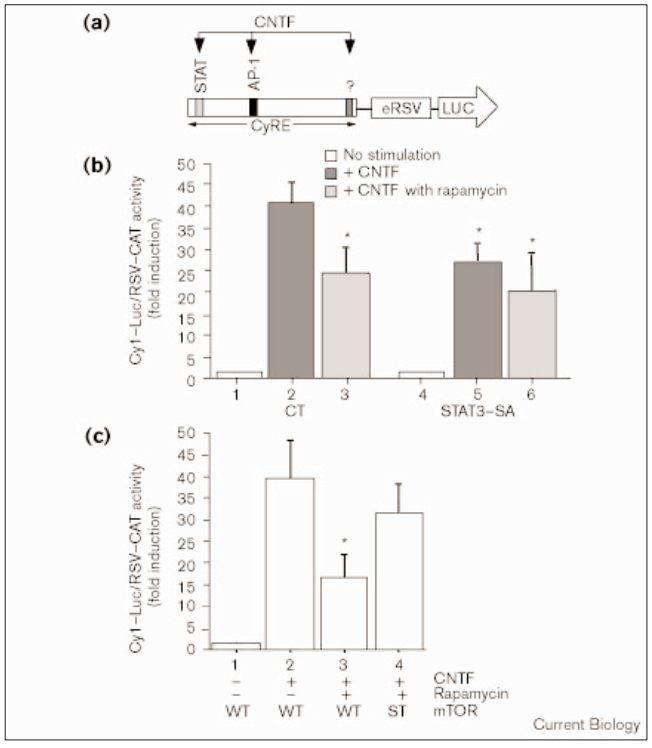
We next used a STAT-responsive luciferase reporter (Cy1–Luc) containing the 180 base pair (bp) CyRE region of the human VIP gene to examine the functional significance of mTOR-mediated serine phosphorylation of STAT3 (Figure 3a). Within the CyRE, two motifs have been characterized that bind transcriptional complexes made up of STAT dimers and AP-1 (composed of c-Fos and JunB or JunD) [11] after CNTF treatment. A third element contributing to full activation has recently been identified at the 3′ end of the CyRE (A. Symes, personal communication). All three motifs are required to achieve maximal CNTF-induced transcription [11]. Transfection of NBFL cells with the Cy1–Luc reporter followed by CNTF

Figure 2



In vitro phosphorylation of STAT3 at Ser727 by mTOR. **(a)** mTOR-WT immunoprecipitated from CNTF-stimulated cells (black bars) was used in an *in vitro* kinase assay with the indicated substrates. Data points were performed in triplicate and are expressed as the mean ± SD. **(b)** mTOR-WT or mTOR-NK immunoprecipitated from CNTF-stimulated cells (black bars) were used in an *in vitro* kinase assay with STAT3 peptide as a substrate. **(c)** p70 S6 kinase immunoprecipitated from CNTF-stimulated cells (black bars) was used in *in vitro* kinase assays with the indicated substrates. White bars in (a–c) represent kinase activity immunoprecipitated from unstimulated cells. *$p < 0.05$ compared with kinase activity from cells not stimulated with CNTF.

Figure 3



CNTF-induced mTOR activation and Ser727 phosphorylation are required for maximal activation of a STAT3-responsive reporter. **(a)** Diagram of the STAT-responsive luciferase reporter construct (Cy1–Luc) containing the 180 bp CyRE region of the human VIP gene. The three elements indicated within the CyRE are required for full induction of the reporter by CNTF (see text for details). **(b)** NBFL cells were co-transfected with Cy1–Luc, and either an empty expression vector (CT) or STAT3-SA. A pRSV–CAT (chloramphenicol acetyltransferase) reporter construct was used as an internal control of transfection efficiencies. Transfected cells were untreated or pretreated with 5 ng/ml rapamycin for 1 h prior to stimulation with CNTF. Luciferase activities were normalized to CAT activities. *$p < 0.05$ versus mock-transfected cells with CNTF stimulation (lane 2). **(c)** NBFL cells were co-transfected with Cy1–Luc, pRSV–CAT, and either HA–mTOR-WT or a rapamycin-resistant mutant HA–mTOR-ST. *$p < 0.05$ versus mTOR-WT-transfected cells (lane 2). In all transfections, results are the mean ± SD of three independent experiments performed in duplicate.

stimulation resulted in a ~40-fold increase in luciferase activity compared with unstimulated cells (Figure 3b, lanes 1,2); rapamycin pretreatment of the transfected cells decreased the CNTF-stimulated increase in luciferase activity to ~25-fold (Figure 3b, lane 3). Similarly, when a STAT3 Ser727Ala mutant (STAT3-SA) was co-transfected along with the Cy1–Luc reporter into NBFL cells, the CNTF-stimulated increase in luciferase activity was also decreased to ~25-fold (Figure 3b, lane 5). Pretreatment of the STAT3-SA/Cy1–Luc transfected cells with rapamycin did not result in a further decrease in the CNTF-stimulated induction of luciferase activity (Figure 3b, lane 6). We confirmed that the concentration of rapamycin used in our experiments specifically inhibited CNTF-stimulated mTOR activity by transfecting NBFL cells with either mTOR-WT or with a rapamycin-resistant mTOR mutant (mTOR-ST) [12] in the absence or presence of rapamycin (Figure 3c). As expected, in cells co-transfected with Cy1–Luc and mTOR-WT, CNTF stimulated a ~40-fold increase in luciferase activity; rapamycin pretreatment decreased the stimulatory effect to ~18-fold (Figure 3c, lanes 1–3). However, in cells co-transfected with the rapamycin-resistant mTOR-ST and pretreated with rapamycin, CNTF-stimulated activation of Cy1–Luc was restored to that observed in CNTF-stimulated cells not pretreated (Figure 3c, lane 4). Thus these data provide strong evidence that the protein kinase activated by CNTF and which mediates phosphorylation of STAT3 at Ser727 is mTOR.

In this study we identified mTOR as the serine kinase that is necessary for full transcriptional activation of STAT3 in CNTF-stimulated cells. mTOR (also known as FRAP, RAFT or RAPT) is the mammalian homologue of the yeast TOR1 and TOR2 proteins [9] and belongs to a family of phosphatidylinositol kinase-related kinases that includes ATM, MEC1, TEL1, RAD3 and MEI-41. Members of this family have been shown to be involved in a range of essential cell functions in yeast and mammalian cells, including cell-cycle progression, DNA repair and DNA recombination [15]. Our data show that mTOR phosphorylates STAT3 at Ser727 in a CNTF-dependent manner in NBFL and HEK293T cells and that this serine phosphorylation is required for STAT3 to maximally activate transcription of a target gene. For

cytokines and growth factors to promote phenotypic changes in mammalian cells they must coordinate activation of transcription with activation of the translation apparatus. The ability of mTOR to contribute to activation of key components of the translation apparatus, p70 S6 kinase and 4E-BP1, as well as the transcription factor STAT3, is an example of such coordination. Interestingly, it has been reported recently that protein phosphatase 2A (PP2A) can regulate phosphorylation of STAT3 on Ser727 [16], although it was not determined whether PP2A accomplishes this directly or indirectly. It is not likely that PP2A can directly dephosphorylate STAT3 in our system, however, as we included a potent inhibitor of PP2A (microcystin LR) in our mTOR kinase assay. In addition, although there are reports that PP2A and mTOR may be in the same pathway, it is controversial whether PP2A is located upstream or downstream of mTOR [17,18]. Further studies, therefore, are currently underway to clarify these findings with regard to serine phosphorylation of STAT3.

Materials and methods

Cell culture and transfection

NBFL cells and HEK293T cells were transfected with Cy1–Luc, pRcCMV/STAT3 mutant SA (Ser727Ala) [4] (kindly provided by J. Darnell), pCDNA1/HA-tagged wild-type mTOR, kinase-dead mTOR mutant NK (Asn2343Lys) or the rapamycin-resistant mutant ST (Ser2035Thr) [12], and pRSV–CAT. Transfected NBFL cells were placed in serum-free medium for 24 h and then pretreated with rapamycin and stimulated with CNTF (25 ng/ml). Lysates were prepared 12–24 h later and luciferase and CAT activities were assayed [2].

Cell lysis and immunoblotting

Standard procedures were used (see Supplementary material).

p70 S6 kinase assay

Assays were performed using a p70 S6 Kinase Assay kit (UBI, Lake Placid, NY) according to the manufacturer's recommendations. Substrates used in the assays were S6 kinase substrate peptide (Ala–Lys–Arg–Arg–Arg–Leu–Ser–Ser–Leu–Arg–Ala) or STAT3(720–731) peptide (Asn–Thr–Ile–Asp–Leu–Pro–Met–Ser–Pro–Arg–Thr–Leu).

mTOR kinase assay

Assays were performed essentially as described previously [19]. 293T cells were transfected with HA-tagged WT or NK mTOR [12], placed in serum-free medium for 24 h and then stimulated with CNTF (25 ng/ml). Lysates were precleared and immunoprecipitated with anti-HA antibody. Immunoprecipitates were then used in mTOR kinase assay with the following substrates: STAT3(720–731) peptide, PKC substrate peptide (Glu–Lys–Arg–Pro–Ser–Gln–Arg–Ser–Lys–Tyr–Leu) and GST–4E-BP1 protein.

Supplementary material

Supplementary material including experimental details of cell lysis and immunoblotting is available at http://current-biology.com/supmat/supmatin.htm.

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