3 Discussion

3.1 Identification of the TOS motif

mTOR functions as a central regulator of cell growth, however, the molecular basis for its activation and its ability to signal downstream are only poorly understood (Gingras, Raught et al. 2001). Different mechanisms for the regulation of S6 kinases and 4E-BPs by mTOR have been suggested. *In vitro* phosphorylation of S6K1 and 4E-BP1 by immunoprecipitated mTOR implies that mTOR directly phosphorylates these substrates. The lack of a mTOR consensus phosphorylation motif present in S6K1 and 4E-BP1, however, has questioned this model. An alternative model suggests that mTOR may rather regulate S6K1 and 4E-BP1 phosphorylation by activating an associated kinase or inhibiting a phosphatase (Dufner and Thomas 1999; Gingras, Raught et al. 1999; Isotani, Hara et al. 1999; Sabatini, Barrow et al. 1999).

The aim of this study was to elucidate the molecular mechanism by which mTOR regulates its downstream targets, S6K1 and 4E-BP1. This work has led to the identification of a conserved motif in the N-terminus of S6K1 and in the C-terminus of 4E-BP1 that is crucial for their phosphorylation by the mTOR pathway. This sequence is designated here as the TOR signaling motif, or TOS motif. Mutations in the TOS motif affect phosphorylation of several sites in S6K1 and 4E-BP1 resulting in inactivation of their functions, reminiscent of the effect of rapamycin.

The initial focus was on the regulation of S6K1 by its TOS motif. The results indicated that the TOS motif mediates mTOR-dependent S6K1 phosphorylation by at least two mechanisms: direct phosphorylation of the hydrophobic motif site Thr389 (Figure 2.1.2 and 2.1.4B) and suppression of an inhibitory effect mediated by the S6K1 C-terminus (Figure 2.1.3 and 2.1.5B). A conserved sequence in the C-terminus of S6K1 has also been identified that may mediate this inhibitory effect (Figure 2.1.8A-D). Deletion of the conserved C-

terminal RSPRR motif partially rescued the inhibitory effect of a TOS motif mutant and rendered S6K1 partially rapamycin-resistant (Figure 2.1.10B). The TOS motif specifically mediates mTOR signaling towards S6K1 by the following criteria: First, the TOS motif is essential for S6K1 activation by mTOR, as mutations in this motif mimicked the effect of rapamycin on S6K1 phosphorylation (Figure 2.1.2B). Second, only overexpression of S6K1 constructs with an intact TOS motif prevented 4E-BP1 phosphorylation by a common mTOR-regulated modulator of S6K1 and 4E-BP1 (Figure 2.1.6). Third, mutations in the TOS motif rendered S6K1 insensitive to amino acid-dependent mTOR signaling (Figure 2.1.7).

In the second part of this research, the mechanism of how the TOS motif in 4E-BP1 mediates mTOR signaling was examined. The TOS motif in 4E-BP1 was found to be absolutely required for efficient *in vivo* phosphorylation of 4E-BP1 at all the identified mTOR-regulated sites, namely Thr37/46, Ser65, and Thr70 (Figure 2.2.1C-E). Furthermore, the mechanism by which the TOS motif in 4E-BP1 mediates mTOR signaling was elucidated. The 4E-BP1 TOS motif is required for 4E-BP1 to bind to the mTOR regulator raptor (Figure 2.2.2A and 2.2.2B), and to be efficiently phosphorylated by the mTOR/raptor complex *in vitro* (Figure 2.2.3A and 2.2.3B). In addition, mTOR signaling via the TOS motif was shown to be important for the complete insulin-stimulated release of 4E-BP1 from eIF4E, which is a prerequisite for proper cell growth control (Figure 2.2.4). As expected, expression of 4E-BP1 containing a mutated TOS motif reduced cell size (Figure 2.2.5A and 2.2.5B).

3.1.1 Regulation of S6K1 phosphorylation by the TOS motif

Mutating the S6K1 TOS motif (F5A) dramatically reduced S6K1 kinase activity and phosphorylation at Thr389 (Figure 2.1.1C and 2.1.2), whereas mimicking of Thr389 phosphorylation partially overcame the inhibitory effect of the TOS motif mutant on S6K1 kinase activity (Figure 2.1.4B). These data indicate that the TOS

motif is required for S6K1 phosphorylation at Thr389. Phosphorylation of Thr389 has been suggested to be regulated by TOR signaling, since phosphorylation of this site is stimulated by amino acids and inhibited by rapamycin (Pullen and Thomas 1997). *In vitro* phosphorylation of Thr389 by mTOR suggests that Thr389 might be directly phosphorylated by mTOR *in vivo* (Burnett, Barrow et al. 1998; Isotani, Hara et al. 1999).

Mutation of the Thr389 site to an acidic residue only partially rescued the activity of the TOS motif mutant S6K1-F5A (Figure 2.1.4B), indicating that Thr389 phosphorylation is not the only TOS-motif mediated input for S6K1 activation. Only a combination of mimicking Thr389 phosphorylation and deletion of the C-terminal domain (F5A-E389ΔCT) completely overcame the inhibitory effect of the F5A mutation, and rendered this S6K1 mutant fully active and rapamycin-resistant (Figure 2.1.5B). These data suggest that a second C-terminal-mediated regulatory mechanism requires the TOS motif to be suppressed. mTOR might suppress the autoinhibitory function of the C-terminus or inhibit the activity of a C-terminal associated phosphatase. The rapamycin-sensitive dephosphorylation of diverse sites in S6K1, such as Thr229(Phe), Thr389(Tyr) and Ser404(Phe) (Figure 2.1.2B) (Pearson, Dennis et al. 1995; Weng, Andrabi et al. 1995) is consistent with the activation of a relatively sequence nonspecific PP2A-like phosphatase. Therefore, in addition to a direct input, mTOR might regulate its targets by inhibiting a phosphatase that interacts with the C-terminus and is capable to dephosphorylating several phosphorylation sites in S6K1. This would allow for a dynamic regulation of S6K1 activity in response to nutrients and mitogenic agonists. If a phosphatase binds to the C-terminus, a higher basal activity of mutants with C-terminal truncations would be expected. S6K1-E389ΔCT and F5AE389ΔCT have higher basal activities compared to their insulin-stimulated activities and the insulin-stimulated activity of wild type S6K1 (Figure 2.1.5B). S6K1-ΔCT does not have a higher basal activity than the wild type enzyme (Figure 2.1.3C), suggesting that Thr389 phosphorylation still requires

a mitogen-regulated input. The basal activity of S6K1-ΔCT, however, decreases more slowly than the basal activity of wild type S6K1 upon amino acid withdrawal, consistent with the requirement of a mTOR-regulated phosphatase binding at the C-terminus (data not shown). A conserved C-terminal motif (RSPRR) may mediate this inhibitory effect of the S6K1 C-terminus, as deletion of this RSPRR motif rendered S6K1 rapamycin-resistant, whereas S6K1 truncation mutants that still contained the RSPRR motif were completely rapamycin-sensitive (Figure 2.1.8A and 2.1.8D). Importantly, the RSPRR motif cooperated with the N-terminal TOS motif in regulating S6K1, as deletion of the RSPRR partially rescued the activity of the F5A TOS motif mutant (Figure 2.1.10B). Thus, the RSPRR motif may be a binding site for a mTOR-regulated phosphatase.

Another model on how a mTOR-regulated phosphatase might regulate S6K1 was based on the observation that S6K1-ΔCT is mitogen-regulated and still rapamycin-sensitive, whereas S6K1-ΔNT/CT is not rapamycin-sensitive. This model suggested that a PP2A like phosphatase, that is inhibited by mTOR, would bind to the N-terminus and dephosphorylate S6K1 (Weng, Andrabi et al. 1995; Dennis, Pullen et al. 1996). However, this model does not take into account the fact that S6K1-ΔCT is partially rapamycin-resistant and that its activity in the presence of rapamycin is comparable to the mitogen-stimulated activity of S6K1-ΔNT/CT (Figure 2.1.3C). Furthermore, these previous experiments did not address the possibility that a phosphatase interacts with the C-terminus of S6K1. The data presented here rather suggest that the deletion of the S6K1 C-terminus, in particular deletion of the RSPRR motif, contribute to the observed rapamycin resistance (Figure 2.1.3C and 2.1.8A and 2.1.8D).

Another possible mechanism for mTOR to regulate the C-terminal inhibitory pseudosubstrate domain of S6K1 is through its phosphorylation. Mutating the rapamycin sensitive site Ser404 to Asp (Figure 2.1.8C) or

substituting all of the other known phosphorylation sites in the C-terminus to acidic residues (S6K1-D_3E) did not change the rapamycin sensitivity of the S6K1 activity (Cheatham, Monfar et al. 1995; Pearson, Dennis et al. 1995 and data not shown). These findings suggest that the mTOR input mediated by the C-terminus of S6K1 is not regulated by phosphorylation of known sites but more likely regulated by another mechanism that might involve a phosphatase.

3.1.2 Regulation of 4E-BP1 by the TOS motif

Similar to S6K1, the TOS motif in 4E-BP1 is required for phosphorylation of several sites in 4E-BP1. The high basal phosphorylation of the 'priming' sites Thr37/46 in serum-starved cells was significantly reduced in the 4E-BP1-F114A mutant (Figure 2.2.1C and 2.2.1D), indicating that the TOS motif is absolutely required for Thr37/46 phosphorylation under serum-starved conditions. Thr37/46 may be directly phosphorylated by mTOR under serum-starved but nutrient-rich conditions, given that Thr37/46 are phosphorylated by mTOR-immunoprecipitates *in vitro* (Brunn, Fadden et al. 1997; Burnett, Barrow et al. 1998; Heesom and Denton 1999). The residual insulin-stimulated phosphorylation on 4E-BP1-F114A could be mediated by an insulin/mitogen-stimulated kinase that phosphorylates Thr37/46 independently of the TOS motif (Figure 2.2.1C and 2.2.1D). The existence of a serum-stimulated, mTOR-independent kinase for Thr37/46 has been previously suggested (Gingras, Gygi et al. 1999).

In contrast, phosphorylation of Ser65 and Thr70 appears to more dependent on mTOR signaling under mitogen-stimulated conditions. Phosphorylation of Ser65 and Thr70 in the TOS motif mutant (4E-BP1-F114A) was more significantly reduced under insulin-stimulated conditions (Figure 2.2.1C and 2.2.1D). Mimicking Thr37/46 phosphorylation with acidic amino acid substitutions did not rescue Ser65 and Thr70 phosphorylation in the 4E-BP1-F114A mutant (Figure 2.2.1E), demonstrating that the TOS motif's effect on Ser65 and Thr70 is not merely a secondary consequence of its effect on Thr37/46

phosphorylation. Ser65 and Thr70 are not likely directly phosphorylated by mTOR, given that mTOR does not phosphorylate these sites in *vitro* (Burnett, Barrow et al. 1998). Phosphorylation of Ser65 and Thr70 has been suggested to be regulated by a mTOR-regulated kinase (Heesom and Denton 1999) and/or by a mTOR-regulated phosphatase (Lin and Lawrence 1997).

During the course of this project, the mTOR binding partner and regulator raptor was identified (Hara, Maruki et al. 2002; Kim, Sarbassov et al. 2002). Raptor also interacts with 4E-BP1 and S6K1, and raptor's association with mTOR enhances mTOR *in vitro* kinase activity towards S6K1 and 4E-BP1 (Hara, Maruki et al. 2002). Reduction of raptor levels by siRNA causes a decrease of 4E-BP1 and S6K1 phosphorylation, indicating that raptor is crucial for phosphorylation of 4E-BP1 and S6K1 *in vivo* (Kim, Sarbassov et al. 2002). Thus, raptor has been suggested to function as a scaffolding protein that recruits mTOR to its substrates, S6K1 and 4E-BP1 (Hara, Maruki et al. 2002).

The 4E-BP1 TOS motif was shown to be absolutely required for 4E-BP's binding to raptor, as only 4E-BP1 wild-type but not the TOS motif 4E-BP1 mutant (4E-BP1-F114A) co-immunoprecipitated with raptor (Figure 2.2.2A and 2.2.2B). In addition, it was demonstrated that 4E-BP1 requires a functional TOS motif for its efficient *in vitro* phosphorylation by the mTOR/raptor complex (Figure 2.2.3A). Based on previous studies and the data presented here, the following model of TOS motif-mediated regulation of 4E-BP1 is proposed (Figure 3.1): the TOS motif mediates 4E-BP1 binding to raptor, which in turn recruits mTOR to the raptor/4E-BP1 complex. 4E-BP1 might directly bind to raptor or the binding may be mediated by unidentified components within the mTOR complex. This recruitment of mTOR enables direct and/or indirect phosphorylation of 4E-BP1 at Thr37/46, Ser65 and Thr70. Under amino-acid rich but serum-starved conditions, mTOR likely forms a constitutively active complex with raptor that directly phosphorylates 4E-BP1 at the Thr37/46 priming sites to allow for additional mitogen-stimulated phosphorylation (see model: Figure 3.1).

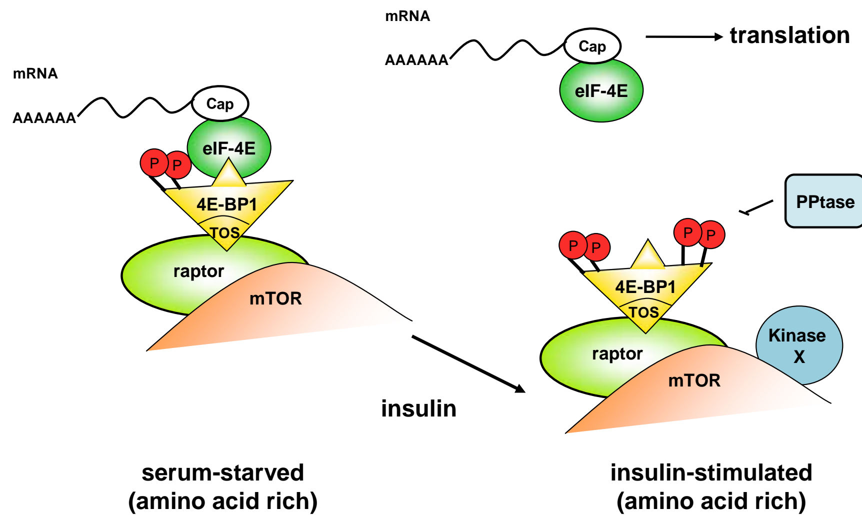

Figure 3.1. **Model of 4E-BP1 regulation by mTOR signaling.** The mTOR/raptor/4E-BP1 complex exists in two forms under amino acid rich conditions. In serum-starved cells, mTOR directly phosphorylates the 4E-BP1 priming sites Thr37/46. Insulin/serum stimulation leads to phosphorylation of Ser65 and Thr70 by a mTOR-regulated kinase (kinase X) and/or inhibition of dephosphorylation of these sites by a mTOR-regulated phosphatase (PPtase). Phosphorylation of Thr37/46, Ser65 and Thr70 causes dissociation of eIF4E from 4E-BP1.

A weak binding of wild-type S6K1 to raptor was also detected (data not shown). Unfortunately, this weak interaction made it experimentally difficult to further characterize the contribution of the S6K1 TOS motif to raptor binding. The difference in raptor binding affinity to S6K1 when compared to 4E-BP1could be caused by a difference of composition of S6K1- or 4E-BP1-raptor-mTOR complexes, with additional molecules found specifically in one or the other complex, or as a result of the existence of additional binding determinants in 4E-BP1.

In complementary work, two groups reported that the TOS motif in 4E-BP1 is required for 4E-BP1 to bind to raptor and to be efficiently *in vitro* phosphorylated by the raptor/mTOR complex. Interestingly, Nojima *et al.*

(Nojima, Tokunaga et al. 2003) found that S6K1 also binds to raptor via its TOS motif. However, phosphorylation of S6K1 by mTOR *in vitro* does not absolutely require a functional TOS motif, as it does for 4E-BP1. The S6K1 TOS motif is only required for raptor-stimulated S6K1 phosphorylation by mTOR *in vitro*.

In addition, Choi *et al.* (Choi, McMahon et al. 2003) demonstrated that the RAIP motif, another conserved motif in 4E-BP1, is required for 4E-BP1 to bind to raptor and to be efficiently phosphorylated by mTOR immunoprecipitates. The RAIP motif is located in the N-terminus of 4E-BP1 and 4E-BP2, but not found in 4E-BP3. The RAIP motif was first identified as a motif that is required for caspase cleavage of 4E-BP1 during apoptosis and is critical for 4E-BP1 phosphorylation on its mTOR-regulated sites (Tee and Proud 2002). Thus, 4E-BP1 binding to raptor may be mediated by two conserved motives, the TOS and the RAIP motif.

In addition to KOG-1 (S.cerevisiae homolog of raptor), LST8, another potential scaffolding protein, was identified as a component of the rapamycin-sensitive TOR complex in *S. cerevisiae* (Loewith, Jacinto et al. 2002). LST8 consists almost entirely of seven WD40 repeats, which often function as scaffolds in multimeric complexes. mLST8 or GβL, the human homolog of LST8, binds to both raptor and mTOR and renders the interaction between raptor and mTOR sensitive to nutrients (Kim, Sarbassov dos et al. 2003). mLST8/GβL may mediate the nutrient-dependent activation of mTOR by raptor. However, the binding of GβL to mTOR itself is not sensitive to nutrients, rapamycin, or detergents, suggesting that mLST8/GβL may be constitutively associated with mTOR. Knocking down protein expression of mLST8/GβL by siRNA decreases S6K1 phosphorylation and phosphorylation of the ribosomal S6 protein, indicating that mLST8/ GβL is required for TOR regulation of downstream signaling to 4E-BP1 and S6K1. It will be interesting to determine whether mLST8/GβL directly binds to 4E-BP1 and/or S6K1 and whether mLST8/GβL is required for 4E-BP1 and S6K1 to bind to the raptor/mTOR complex. mLST8/GβL may have different

binding affinities for 4E-BP1 or S6K1, and the different binding affinities might explain why 4E-BP1 binds stronger to the mTOR/raptor complex than S6K1.

4E-BP1 functions to regulate cell growth/cell size and cellular proliferation (Miron and Sonenberg 2001; Fingar, Salama et al. 2002). Overexpression of 4E-BP1 antagonizes transformation induced by eIF4E overexpression (Rousseau, Gingras et al. 1996), and overexpression of dominant negative 4E-BP1 mutants in cycling mammalian U2OS cells (Fingar, Salama et al. 2002) or *Drosophila* (Miron and Sonenberg 2001) reduces cell size. The data presented here demonstrate that 4E-BP1 regulation by mTOR via the TOS motif is required for 4E-BP1-mediated control of cell size. Overexpression of 4E-BP1-F114A reduced the size of G_1-phase gated U2OS cells similar to the dominant negative 4E-BP1mutant 4E-BP1-T37/46A (Figure 2.2.5A and 2.2.5B). The effect of 4E-BP1-F114A overexpression on cell growth is consistent with the lack of 4E-BP1 phosphorylation by mTOR via the TOS motif, as several of these phosphorylation events are required to release eIF4E and promote cap-dependent translation (Mothe-Satney, Brunn et al. 2000; Gingras, Raught et al. 2001). Mutation in the eIF4E-binding site on 4E-BP1-F114A (4E-BP1-F114A-Y54A/L59A), although still hypophosphorylated, abrogated its ability to bind to eIF4E and to reduce cell size (Figure 2.2.5B). Thus, the growth inhibitory effect of 4E-BP1-F114A is mediated via its constitutive binding to and inhibition of eIF4E. Previously, our laboratory provided evidence that mTOR independently signals to both S6K1 and 4E-BP1/eIF4E to control cell size (Fingar, Salama et al. 2002). Increased S6K1 activity and eIF4E expression increase cell size in both the presence and absence of rapamycin, individually and additively, indicating that S6K1 and eIF4E indeed are important regulators of cell size. But signaling from mTOR to S6K1 and eIF4E is not sufficient to restore cell size and proliferation during rapamycin treatment, suggesting a novel mTOR pathway(s) is important for cell size control.

3.1.3 Conclusions

The data presented here and previous observations (Hara, Maruki et al. 2002; Choi, McMahon et al. 2003; Nojima, Tokunaga et al. 2003) support the model that mTOR directly phosphorylates 4E-BP1 at Thr37 and Thr46 and S6K1 at Thr389. However, mTOR phosphorylates S6K1 and 4E-BP1 very poorly on its own and requires the scaffolding protein raptor to recruit S6K1 and 4E-BP1 into its close proximity for efficient phosphorylation of these substrates. Thus, the substrate specificity of mTOR is defined through the specific recognition of its substrates by the scaffolding protein and not only through a consensus phosphorylation motif in the substrates, as it is often found for kinases. The requirement of a scaffolding protein to determine substrate specificity of mTOR may allow an additional level of mTOR regulation. Different scaffolding proteins may recruit different groups of substrates to mTOR and the recruitment may be dependent on the cellular conditions. Raptor may target TOS motif-containing substrates to mTOR, whereas other potential mTOR scaffolding proteins may recruit a different class of substrates that contain a different recognition motif. Interestingly, the association of raptor with mTOR is sensitive to changes in amino acid levels (Kim, Sarbassov et al. 2002), suggesting that raptor selectively recruits substrates only under amino acid rich conditions. Consistently, mTOR-dependent phosphorylation of S6K1 and 4E-BP1 is sensitive to amino acids.

Not all of the mTOR-dependent phosphorylation sites in S6K1 and 4E-BP1 are likely to be directly phosphorylated by mTOR. mTOR is not capable of directly phosphorylating Ser65 and Thr70 in 4E-BP1 *in vitro* (Burnett, Barrow et al. 1998), however, phosphorylation of Ser65 and Thr70 *in vivo* requires a functional TOS-motif (Figure 2.2.1C and 2.2.1D). Therefore, the kinase acting on these sites may also be located in the mTOR/raptor complex. In addition, the C-terminal inhibitory effect in S6K1 might be caused by a phosphatase that binds to the S6K1 C-terminus and is repressed by mTOR. Thus, the TOS motif in S6K1

and 4E-BP1 is a docking site for the raptor/mTOR complex which likely regulates phosphorylation of different sites in S6K1 and 4E-BP1 by different mechanisms.

Importantly, the regulation of 4E-BP1 phosphorylation by the TOS motif is biologically significant: expression of 4E-BP1-F114A reduces cell size, indicating that the TOS motif is crucial for regulation of cell growth by raptor/mTOR signaling. The TOS motif appears to function as a general recognition site that allows raptor to bring downstream substrates to mTOR. In the future it is expected to identify novel mTOR substrates using our mechanistic knowledge of the TOS motif.

3.2 Regulation of the mTOR target S6K2

Similar to S6K1, S6K2 is regulated by the PI3K and mTOR pathway. During cell treatments that potentially activate S6K1, S6K2 is significantly less activated (Martin, Schalm et al. 2001 and Figure 2.3.3). Therefore, activators specific for S6K2 may be required to release an additional inhibitory effect. The identification of activators specific for S6K2 might help to better understand the physiological function of S6K2. Deletion of the C-terminus of S6K2 increases its kinase activity, suggesting that the C-terminus of S6K2 mediates an inhibitory effect (Martin, Schalm et al. 2001). A proline rich region only found in the C-terminus of S6K2 but not in S6K1 is a good candidate domain that may mediate this inhibitory effect. Proline-rich domains often bind to SH3 domains of other proteins. Therefore, the proline rich domain of S6K2 may bind to an activator that possesses a SH3 domain.

However, replacing the proline-rich domain of S6K2 by the corresponding part of S6K1 did not significantly affect the insulin-stimulated activity of S6K2 (Figure 2.3.2B). Therefore, the proline rich domain of S6K2 seems to be not responsible for the additional inhibition of S6K2 kinase activity. The proline-rich domain might rather be required for S6K2 to signal to substrates and/or control the

sub-cellular localization of S6K2. Further experiments will be necessary to determine the role of this proline-rich domain.

In contrast, replacing the N- or C-terminus of S6K2 by the corresponding parts of S6K1 significantly increased S6K2's insulin-stimulated activity (Figure 2.3.2). In addition, adding the N-or C-terminus of S6K2 onto S6K1 decreased the kinase activity of S6K1 (Figure 2.3.3). These data suggest that the N- and C-terminus of S6K2 mediate an inhibitory effect on S6K2 kinase activity. A phosphatase may bind to the N-terminus of S6K2 and decrease the activity of S6K2 by dephosphorylating S6K2. This would also explain why fusing the N-terminus of S6K2 on S6K1 also decreases the activity of S6K1. Inhibiting the phosphatase activity of PP2A-type phosphatases with Okadaic Acid did not abolish the inhibitory effect mediated by the N-terminus of S6K2, suggesting that the potential phosphatase is not a PP2A-type phosphatase (data not shown). A nuclear localization sequence only found in the extreme C-terminus of S6K2 (Koh, Jee et al. 1999) could be responsible for the inhibitory effect of the C-terminus (Figure 2.3.2 and 2.3.3). Further experiments are required to test these hypothesises.