Review

The role of protein synthesis in cell cycling and cancer

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ABSTRACT

Cell cycling and protein synthesis are both key physiological tasks for cancer cells. Here we present a model for how the elongation phase of protein synthesis, governed by elongation factor 2 and elongation factor 2 kinase, both modulates and responds to cell cycling. Within this framework we also discuss survivin, a protein with both pro-mitotic and anti-apoptotic roles whose persistence in the cell is tied to protein synthesis due to its short half-life. Finally, we provide a brief overview of efforts of cancer researchers to target EF2 and EF2 kinase.

1. Introduction

Protein synthesis is a finely tuned and tightly controlled process. The elongation step of this process has attracted the attention of cancer researchers. In particular, elongation factor 2 (EF2), the critical enzyme governing elongation of nascent proteins, has been investigated as a target for new therapies and as a potential contributor to the success of conventional therapies.

EF2 is solely responsible for the translocation of codons from the A to P ribosomal positions. Upon EF2 inactivation by phosphorylation or ADP ribosylation, protein synthesis is halted with mRNA-loaded ribosomes primed to resume protein synthesis once EF2 is freed from the inactivating influence (Sivan et al., 2007). ADP ribosylation of EF2 is generally the result of an exogenous toxin. The diphtheria toxin, and Pseudomonas aeruginosa extoxin A have both been shown to be culprits (Jorgensen et al., 2006; Fitzgerald and Pastan, 1993). Most commonly, however, the inactivation of EF2 is achieved via phosphorylation of Thr56 by EF2 kinase, a dedicated kinase with EF2 as the only known substrate (Ryazanov and Spirin, 1990). Phosphorylation of EF2 precludes functional binding to the ribosome, thereby stalling the elongation of nascent proteins (Proud, 2007). This internal mode of control is important for healthy function but can also, if dysregulated, be a factor in disease.

EF2 and EF2 kinase have an inverse relationship inasmuch as when one enzyme is active, the other is necessarily inactive. This is clearly demonstrated under cellular metabolic stress such as starvation. Such conditions activate AMPK, which in turn activates EF2 kinase (Browne et al., 2004). When EF2 kinase then phosphorylates EF2, protein synthesis is subsequently inhibited, a reasonable survival strategy when conservation of resources is critical (Figure 1).

Interestingly for cancer researchers, EF2 and EF2 kinase also follow a prescribed pattern during the progression of the cell cycle (Table 1). This review will provide a concise summary of the interplay between EF2 and each phase of the cell cycle. It is certainly a reciprocal relationship, such that EF2 is influenced by cell cycle progression and also influences cell cycle progression. The specific importance of this relationship to understanding and treating cancer will also be discussed.

2. EF2, EF2 kinase, and the cell cycle

2.1. G1-phase

During the G1-phase of the cell cycle protein synthesis is enabled, requiring EF2 activity. Due to the reciprocal relationship of EF2 and EF2 kinase, this capability requires the inactivation of EF2 kinase. The inactivation of EF2 kinase is accomplished through the phosphorylation of the Serine 359 or Serine 366

residues (Proud, 2007) (Figure 2). The phosphorylation of Ser366 is the mechanism of control generally observed during G1, while the role of Ser359 is important during G2/M and will be discussed in connection with that phase of the cell cycle.

The Ser366 site is downstream of both the MKK and mTOR pathways. The mTOR pathway has been extensively studied by Proud and co-workers with a recent review providing an excellent summary (Proud, 2007). The contribution of the MKK pathway to EF2 kinase phosphorylation and the explicit role this plays in cell cycle has recently been examined by Roberts and co-workers who demonstrate that phosphorylation of Ser366 rises quickly upon G1 entry in a synchronized cell population in an MKK-dependent manner (Roberts et al., 2006).

The importance of EF2 activity during G1 has been demonstrated by Gutzkow et al. in a study which examined the interplay between cyclin D3 and EF2 activity. Their work indicated that exogenous activation of PKA results in EF2 kinase activation, EF2 inactivation, and subsequent loss of cyclin D3 in Jurkat cells (Gutzkow et al., 2003). Because cyclin D3 is necessary for progression through G1, cells lacking cyclin D3 arrest in this phase.

2.2. G1/S transition

The entry into S phase requires the activity of EF2 kinase. This activation is not simply the result of dephosphorylation of Ser366. Instead, changes to cellular physiology during this
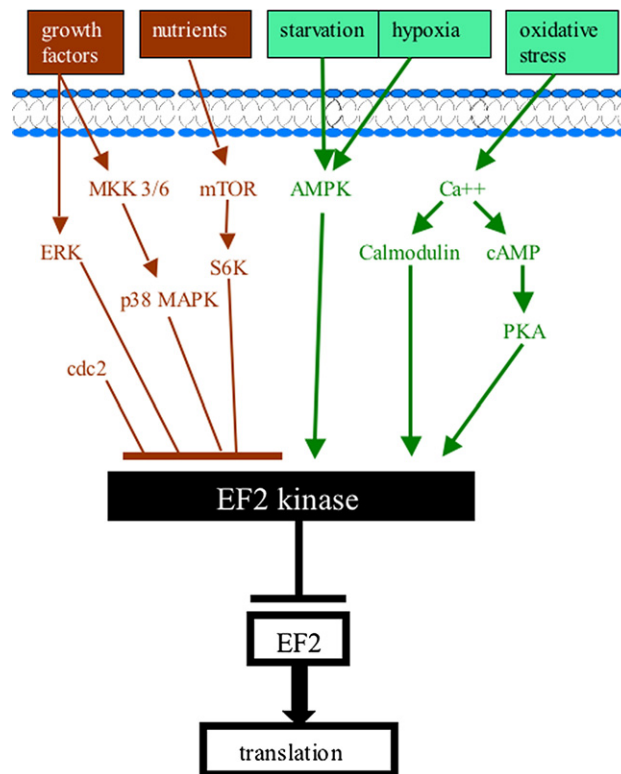
Figure 1 – Upstream pathways that influence EF2 kinase activity. Growth factors and nutrients results in inhibition of EF2 kinase to allow EF2 activity and translation to proceed. In contrast, starvation, hypoxia and oxidative stress stimulate EF2 kinase, which phosphorylates and inactivates EF2 thereby interrupting protein translation. Pathways diagrammed in green depict activation of EF2 kinase, whereas those in red depict inhibition.

Phase of cell cycle	Protein elongation capability	Mechanism of control	Consequence of dysregulation
G1: protein synthesis	EF2 functional	EF2 kinase inactivated by phosphorylation of Ser366	Forced activation of EF2 kinase results in G1 arrest
S: DNA synthesis	EF2 kinase functional	EF2 kinase activated by rise in cAMP and Ca+2 levels	Inhibition of EF2 kinase precludes entry into S phase
G2/M: Proofreading and cell division	EF2 kinase functional	EF2 inactivated by phosphorylated at Thr56	Inactivation of EF2 results in G2/M arrest

Table 1 – Status of protein elongation activity during cell cycling. A summary of cellular protein synthesis at each stage of the cell cycle, the mechanisms that govern the activity, and consequences of dysregulation (see text for details and references).

transition facilitate multiple alterations to EF2 kinase (Figure 2). First, cAMP is strongly upregulated and activates PKA (Saeki et al., 1999; Desdouets et al., 1995). PKA then activates EF2 kinase by phosphorylating Ser500 (Gutzkow et al., 2003; Proud, 2007). This activating phosphorylation could also be influenced by the other major physiological change at G1/S transition: a rise in intracellular Ca^{+2}. Ca^{+2} levels affect a wide range of activities in the cell; one effect is the upregulation of cAMP and, therefore, the upregulation of PKA activity potentially reinforces the aforementioned cAMP activation of PKA (Berridge et al., 2000). However, the PKA phosphorylation of Ser500 has also been shown to induce activation of EF2 kinase independently of calcium (Proud, 2007).

The rise in Ca^{+2} has another well-understood effect on EF2 kinase. In the presence of elevated calcium levels, calmodulin binds to EF2 kinase and strongly activates it (Santella, 1998; Proud, 2007). This binding can be blocked by the phosphorylation of the nearby Ser78 site in an mTOR-mediated manner, thereby blunting the effect of the Ca^{+2} influx (Browne and Proud, 2004). The combination of calmodulin and the phosphorylation of Ser500 during G1/S transitionfunctions to support EF2 kinase activity and thereby suppress EF2 activity during later stages of the cell cycle. Unfortunately, the exact interplay between EF2 and EF2 kinase during S phase proper has yet to be fully characterized. Although strong evidence indicates that EF2 kinase remains activated throughout S phase, the exact timeline for the subsequent inactivation of EF2 is not clear.

2.3. G2/M

During G2/M, the cellular pool of EF2 is phosphorylated by EF2 kinase and protein synthesis drops precipitously (Celis et al., 1990). In addition to the activating effects on EF2 kinase seen at the G1/S transition, by G2/M the phosphorylation at Ser366 which inhibits EF2 kinase during the G1-phase has been removed (Roberts et al., 2006; Sivan et al., 2007).

The decline in protein synthesis during mitosis has been known for decades; publications from the 1960s describe the phenomenon. The most common explanation for this observation is that cellular energy at this time is being directed toward proofreading and the physical division of the mother and daughter cell. Since protein synthesis uses a large percentage of cellular energy during normal metabolism, it is reasonable to propose that during the energy-intensive event of cell division this process is downregulated. Hence, the cell may conserve energy in the same way as it does when under starvation conditions, that being the phosphorylation of EF2.

We have shown that hydrogen peroxide results in phosphorylation of EF2 and accumulation of prostate cancer cells in G2/M (White et al., 2007). In addition, siRNA knockdown of EF2 results in G2/M accumulation in gastric cancer cells (Nakamura et al., 2009). These observations suggest that the inactivation or depletion of EF2 can result in the cell cycling coming to rest in the phase which would ordinarily have little EF2 activity. Unexpectedly, these results contrast with the previously mentioned observation by Gutzkow and colleagues that EF2 inactivation can result in G1 arrest due to loss of cyclin D3. Taken together, the current research suggests that the necessary thresholds of EF2 activity for cell cycle regulation are quite finely tuned (and also may differ between cells) but confirm that EF2 modulation results in alteration to cell cycling.

3.   A closer look at the protein pool during G2/M

This model presents a puzzle for protein synthesis activity during the cell cycle. How does the cell provide for any protein-related needs during the G2/M phase? For instance, proteins with short half-lives generally must be replaced almost continually and some of these proteins, such as survivin, are critical for cell cycle progression during G2/M.

Survivin has been a protein of interest to cancer researchers for many years because it is often overexpressed in cancer cells. Survivin opposes caspase 9 activity, making it a member of the anti-apoptotic family and, by extension, of the oncogene family. Like other anti-apoptotic caspase antagonists such as cFLIP_S and XIAP, survivin has a very short half-life of only 30 min (Zhao et al., 2000). Such a short half-life would predict a disappearance from the cell over the course of G2/M. However, survivin plays a critical role during this time. During mitosis, survivin binds to and stabilizes mitotic spindles (Fortugno et al., 2002). In the absence of survivin, cycling cells undergo mitotic collapse and caspase 9-mediated apoptosis (O'Connor et al., 2000; Yang et al., 2004). Upon mitotic completion, survivin is efficiently removed from the cell by ubiquitination and degradation so that cycling cells harvested in G1 no longer show any evidence of survivin (Zhao et al., 2000). The observation that survivin plays an important role at the end of the G2/M phase seems to contradict the known half-life of survivin as well as the known inhibition of protein synthesis during mitosis. How could such a short half-life protein persist long enough to function during mitosis considering the phosphorylation of EF2 during this time?

The answer to this question is found in the cyclin complex which is active during G2/M: CyclinB and cdc2/CDK1. Cdc2
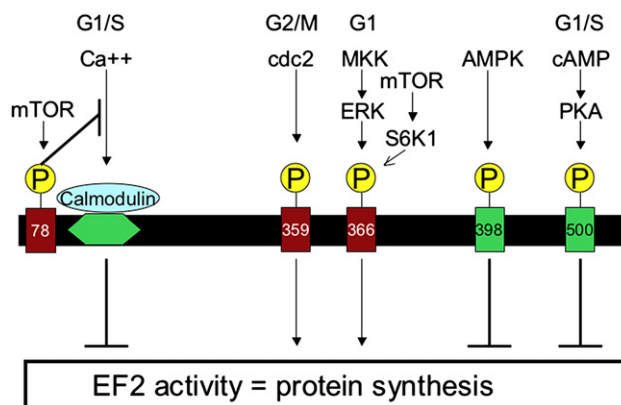
Figure 2 – Schematic of activation/inactivation sites on EF2 kinase. There are currently three described inactivating sites (red) and three described activating sites (green) which are differentially regulated during cell cycle progression. This allows EF2 kinase to respond to complex stimuli and exert control over EF2 activity (details are described in the text).

becomes an active kinase during this phase and makes two important contributions to the protein pool during G2/M. First, cdc2 phosphorylates survivin itself, which stabilizes the protein and lengthens its half-life (Fortugno et al., 2002). If cdc2 is unable to perform this stabilization, the result is a weakening of mitotic spindles due to insufficient survivin (O'Connor et al., 2002). The second contribution to the protein pool has only recently been described by the Proud group and offers a potential loophole for the inhibition of protein synthesis during G2/M. The investigators report that cdc2 is able to deactivate EF2 kinase though phosphorylation at Ser359 (Smith and Proud, 2008). Importantly, phosphorylation at this site inhibits EF2 kinase activity (freeing EF2 to elongate nascent proteins) even in the presence of other activating influences such as Ca^{+2}. Therefore, the activity of cdc2 during G2/M can counteract the other cell cycle influences on EF2 kinase and thereby facilitate protein synthesis during the phase. It should be noted that this effect is only observed when sufficient amino acids are supplied to the cell (Smith and Proud, 2008). It seems that even during cycling, a starvation response to halt protein synthesis will override any other influences on the elongation process (Wu et al., 2006). Taken together, a complex picture of regulation over EF2 kinase and, subsequently, EF2 emerges (Figures 1 and 2).

4.   Dysregulation in cancer

Upregulation of survivin levels in cancer cells has been widely observed, which is not surprising considering the dual role survivin plays as both a pro-mitotic and anti-apoptotic influence (Altieri, 2008; Mita et al., 2008). In some cases, presence of survivin is a diagnostic indicator for cancer. For instance, voided urine may be analyzed for survivin as a sign of bladder cancer and this test is being explored for clinical use (Margulis et al., 2008). A general propensity toward higher levels of anti-apoptotic proteins is not uncommon in cancers, which suggests a possible role for protein synthesis dysregulation in cancer development and growth.

As discussed earlier, anti-apoptotic proteins generally have short half-lives. One consequence of this is that interruption of protein synthesis can quickly result in cellular caspases losing their opposition (White et al., 2007). Cancer cells may be at risk for this because they experience a high level of metabolic stress due to their characteristic metabolic insatiability.

Insatiability poses a variety of problems to the sensitive system governing protein elongation. For instance, cancer cells initially outgrow their blood supply and thereby live under conditions of reduced oxygen and nutrients. Starvation, as previously noted, inhibits protein production through phosphorylation of EF2. Likewise, hypoxia, also mediated by AMPK, can result in phosphorylation of EF2 (Liu et al., 2006) (Figure 1). Even in tumors that are vascularized, thus assuring sufficient nutrients and oxygen, the metabolism of cancer cells predisposes them to unique stresses. For instance, cancer cells tend to limit ATP generation via mitochondrial respiration, instead utilizing the much less efficient glycolytic pathway. This alteration, often referred to as the Warburg effect, may protect cancer cells to some degree from the generation of damaging superoxides and peroxides during respiration (Lopez-Lazaro, 2008). Such a reduction could hypothetically benefit a cancer cell since the presence of free radicals can result in the phosphorylation of EF2 (Figure 1). For example, exposure to hydrogen peroxide results in an increase in intracellular Ca^{+2} levels, thereby activating EF2 kinase and subsequently inhibiting protein synthesis via phosphorylation of EF2 (Kang and Lee, 2001). Because the Warburg effect may limit the generation of free radicals, it may also limit one mechanism of EF2 phosphorylation. However, the anaerobic generation of ATP results in lactic acid as a by-product. The accumulation of lactic acid inevitably lowers the pH of the cell, which also results in the strong activation of EF2 kinase, phosphorylation of EF2, and inhibition of protein synthesis (Dorovkov et al., 2002). Simply stated, maintaining protein synthesis in a metabolically disordered cell is one of the most complicated challenges that cancer cells face.

It seems likely that adaptive cancer cells require a mechanism to absorb the influences which phosphorylate EF2, inhibit protein synthesis, and potentially leave the cell with too few anti-apoptotic proteins. One potential solution is the upregulation of EF2 itself to ensure maintenance of basal enzymatic activity in a stressful environment. Such an upregulation has, indeed, been observed in ovarian, gastric, and colon

cancers (Alaiya et al., 1997; Nakamura et al., 2009). Any observations of this nature are quite impressive because in eukaryotic cells the expression level of EF2 is extremely tightly regulated, making in vitro overexpression of EF2 much more challenging than overexpression of most other proteins (Ortiz and Kinzy, 2005). The ability of cancer cells to achieve upregulation of EF2 appears to be evidence of profound disorder.

Of course, upregulation of EF2 is predicted to come at a cost to a cycling cell. The G1/S phase cannot occur unless EF2 kinase is activated, which implies the requirement for controlled phosphorylation of EF2. Therefore, a rise in EF2 levels could require increased expression of EF2 kinase to cope with a larger substrate burden. Such an upregulation has been repeatedly demonstrated by Hait and co-workers in multiple cancers (Cheng et al., 1995; Parmer et al., 1999; Arora et al., 2005). The overexpression of survivin, EF2, and EF2 kinase seen in cancer, suggests that these proteins fill critical niches in the life of a cancer cell. This observation makes them potential targets for cancer treatments.

5.   Cancer treatments which impact EF2 directly or indirectly

Insights into cell cycle-dependent regulation of EF2 activity have also found applications in the area of cancer therapies. It appears that EF2 plays a part in the mechanism of action of some long-used drugs. Doxorubicin, one of the oldest and most used of the chemotherapeutic arsenal, is pleiotropic but of special significance for this discussion is its recently described effect on EF2 (White et al., 2007). Upon doxorubicin treatment, EF2 is strongly phosphorylated, protein translation is halted, cells arrest in G2/M, and stores of short half-life proteins with anti-apoptotic functions (i.e. cFLIP$_S$, survivin, and XIAP) are quickly depleted, leaving cells more susceptible to induction of cell death. This cascade of effects appears to be dependent on interaction of doxorubicin with intracellular iron and the subsequent generation of free radicals. If cells are pretreated with an iron chelator before doxorubicin treatment, the net effect on the cells is quite minor; anti-apoptotic proteins are preserved and the cells retain an anti-apoptotic phenotype. Conversely, the effects of doxorubicin could be recapitulated by exposure to a low dose of hydrogen peroxide, supporting the idea that free radical generation is an important aspect of doxorubicin therapy. Indeed, the physiological shifts induced when protein synthesis is halted may be an understudied contributor to any number of drug effects since most chemotherapy agents generate free radicals, potentially resulting in the phosphorylation of EF2 (Kong and Lillehei, 1998).

Another commonly used drug to consider is taxol, which is widely considered to act by stabilizing microtubules and thereby arresting cycling cells in the G2/M phase (Huizing et al., 1995). It was recently observed that, in addition, exposure to taxol resulted in the persistent phosphorylation of EF2 by an unknown mechanism (Pineiro et al., 2007). Whether the EF2 phosphorylation is a contributing cause of arrest in G2/M or a result of the arrest is still an open question.

More clear is the observation that EF2 kinase dysfunction is a cause of cell cycle arrest. Several drugs which inhibit EF2 kinase activity including rottlerin, geldanamycin, and the imidazolium histidine kinase inhibitor NH125, have shown that without activation of EF2 kinase the cell cycle arrests at S phase (Arora et al., 2003; Parmer et al., 1999, 1997; Yang et al., 2001). Thus treating EF2 kinase as an explicit target may be a reasonable strategy, yet only NH125 has demonstrable specificity for EF2 kinase (Arora et al., 2003). However, results using this drug are in agreement with the less specific agents, such as geldanamycin. Geldanamycin and its relatives are, more accurately, Hsp90 inhibitors but EF2 kinase is a downstream target because it is chaperoned by Hsp90 (Yang et al., 2001). Without Hsp90, levels of EF2 kinase also drop, presumably below the threshold needed to achieve sufficient activity for S phase. Of course, targeting Hsp90 results in many downstream effects as the protein chaperones a large number of other proteins. In the case of the cell cycle, Hsp90 is particularly important because not only does it chaperone EF2 kinase but also survivin. (Zajac et al., 2008). Thus, upon Hsp90 inhibition survivin levels fall, mitotic spindles are unable to properly form, and cells undergo mitotic collapse (Zajac et al., 2008). The dual downstream impact on both EF2 kinase and survivin results in the potential of a two-pronged arrest. Okamoto and colleagues have shown that in mesothelioma cells treatment with the small molecule Hsp90 inhibitor 17-AAG facilitates accumulation of cells in either G1 (presumably unable to enter S phase without enough EF2 kinase) or in G2/M (presumably unable to perform mitosis without enough survivin) depending on the baseline characteristics of a given cell line (Okamoto et al., 2008).

A relatively new drug, Ontak, is designed to target EF2 directly and has been used successfully to treat several hematological malignancies (Duvic and Talpur, 2008). Ontak is composed of an IL-2 moiety and an inactive form of the diptheria toxin. Once endocytosed by malignant white blood cells overexpressing the IL-2 receptor, the toxin is lysosomally cleaved and activated, allowing it to find and affect its target, EF2 (Nichols et al., 1997). As previously noted, the diphtheria toxin inactivates EF2 via ADP ribosylation, resulting in the inhibition of protein synthesis and eventual death of the cell (Duvic and Talpur, 2008). Such rational drug design is a hopeful indicator that the many lessons learned from an older generation of cancer drugs might be honed and applied with improved specificity and, of course, efficacy. Protein translation may be a fulcrum point in the physiology of a cancer cell, giving cancer researchers a much-needed target.

6.   Conclusions

The elongation stage of protein synthesis, controlled by EF2 and EF2 kinase, may offer clues to the origins and maintenance of cancer cells. The mechanism of protein elongation is closely tied to the progression of the cell cycle, such that dysregulation of EF2/EF2 kinase can alter the cell cycle and vice versa. EF2 or EF2 kinase therefore may be overexpressed in cancer cells in part to modulate their influence over cell cycling. Overexpression would also allow the cellular pools of these enzymes to buffer some of the metabolic stressors that cancer cells inevitably experience, such as hypoxia and the generation of free radicals. We suggest that modifications to the machinery of protein synthesis may indicate the importance of this system in tumorigenesis. We also hope

that cell cycle researchers will be encouraged to investigate EF2 and EF2 kinase within the context of their work. Currently, very little is known about the interactions between these enzymes and the machinery of the cell cycle. Possible relationships with Rb, ubiquitin ligases, and the majority of the cyclin family remain uncharacterized. While decades of cancer research has focused on modifications to DNA, it may also be important to examine how protein production is altered in cancer cells.

Acknowledgements

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