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Minireview

The modular phosphorylation and activation of p70^{s6k}

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Abstract The activation of p70^{s6k} is accompanied by a complex series of phosphorylation events. In this review we propose a model of activation which divides p70^{s6k} into four functional modules that cooperate in leading to full enzyme activity. In the light of the model, we suggest how candidate effectors of p70^{s6k} activation might function by directing the phosphorylation of specific sites.

Key words: Modular phosphorylation; p70^{s6k}

1. The significance of p70^{s6k}/p85^{s6k} in cell cycle progression

The discovery that mitogen-induced metabolic processes maybe regulated by serial phosphorylation and dephosphorylation events has captivated the attention of signal transduction scholars for over a decade [1]. The application of this finding has motivated workers to purify, clone and crystallise many of the proteins responsible for mediating specific mitogenic events. The goal of this laboratory has been to unravel the complex series of signalling events which lead to, and from, the activation of the p70/p85 isoforms of S6 kinase, p70^{s6k}/p85^{s6k} [2–4]. The p70^{s6k} is recognised as the kinase which regulates the multiple phosphorylation of 40S ribosomal protein S6 in vivo, distinct from the p21^{ras}-mediated p90^{rsk} [5]. Inhibition of mitogen-induced p70^{s6k}/p85^{s6k} activation in vivo with either neutralising antibodies [6,7] or by treatment with the immunosuppressant rapamycin [8–10] severely compromises the ability of the cell to progress through the G1 phase of the cell cycle. The importance of these observations is emphasised by the critical role p70^{s6k} plays, presumably through S6 phosphorylation, in regulating the translation of a class of mRNA transcripts which contain an oligopyrimidine tract at their transcriptional start site [4]. This class of mRNAs encode for many of the components of the protein synthetic apparatus and can represent up to 20% of the total mRNA in the cell [11]. Failure to recruit these messages into polysomes suppresses the biogenesis of translational machinery required for cell cycle progression [12].

The p70^{s6k}/p85^{s6k} isoforms are generated from alternative translation start sites on the same transcript (Y. Chen and S. Kozma, unpublished). The two isoforms are coordinately regulated and only differ by a 23 amino acid N-terminal extension which constitutively targets p85^{s6k} to the nucleus [7]. Whilst the mechanism by which p85^{s6k} mediates cell cycle progression is, as yet, unresolved, it is noteworthy that S6 is present in a free form in the nucleus and becomes phosphorylated at the same residues in response to mitogens as its cytoplasmic counterpart [13]. As the role of S6 in the cytoplasm and nucleus has been recently discussed [4,14], we will concentrate here on the upstream signalling elements and the modular mechanisms which culminate in p70^{s6k} activation.

2. The putative signalling components that elevate p70^{s6k} activity

The observation that p70^{s6k} activity could be preserved in cell extracts supplemented with phosphatase inhibitors [15,16] provided the first indication that mitogen-induced kinase activity could be potentiated by a series of phosphorylation events (see below). The kinase was subsequently purified [16,17] and the cDNA cloned [18,19]. MAPK has been shown to directly phosphorylate p70^{s6k} in vitro [20], but studies employing dominant interfering signalling components or receptor mutants [21] have demonstrated that MAPK is neither necessary nor sufficient to elicit p70^{s6k} activation. Indeed, it is becoming increasingly evident that p70^{s6k} activation is a complex process requiring multiple signalling inputs [22–24] and that these inputs are likely to be shared with the p85^{s6k} isoform [7,8]. This complexity has led to the utilisation of two broad approaches in attempting to elucidate the mechanism of activation, with most studies focusing on the p70^{s6k} form.

One approach, which has been in vogue, is to construct a p70^{s6k} signalling pathway by utilising compounds which inhibit p70^{s6k} activation [8,9,25–27] although a full understanding of how these molecules achieve this end is lacking. As shown in the model (Fig. 1) several signalling components have been implicated in the activation of p70^{s6k}. The immunosuppressant rapamycin is the most potent inhibitor of p70^{s6k} described, blocking its activation by all known agents [8,9,25]. The target of rapamycin, as a gain-of-function inhibitory complex with FKBP12, is mTOR/FRAP [28], first identified in yeast as TOR-1/TOR-2 [29]. Since an mTOR/FRAP mutant, which is unable to bind rapamycin-FKBP12, protects p70^{s6k} activity in the presence of the inhibitor, it has been reasoned that mTOR/FRAP is an upstream regulator of p70^{s6k} [30]. mTOR/FRAP is a member of the PIK-related family of protein kinases which contain a C-terminal lipid kinase domain, although no allied lipid kinase activity has been demonstrated yet for any member [31]. Despite the observation that mTOR/FRAP can autophosphorylate [30], other potential protein substrates remain elusive. It is still unclear whether mTOR/FRAP mediates its affects on p70^{s6k} through its kinase domain or through binding an obligatory effector molecule. More importantly, given the intimate relationship between the effects of rapamycin on p70^{s6k} through the N-terminus of the kinase ([24,32,33] and below) it is critical to

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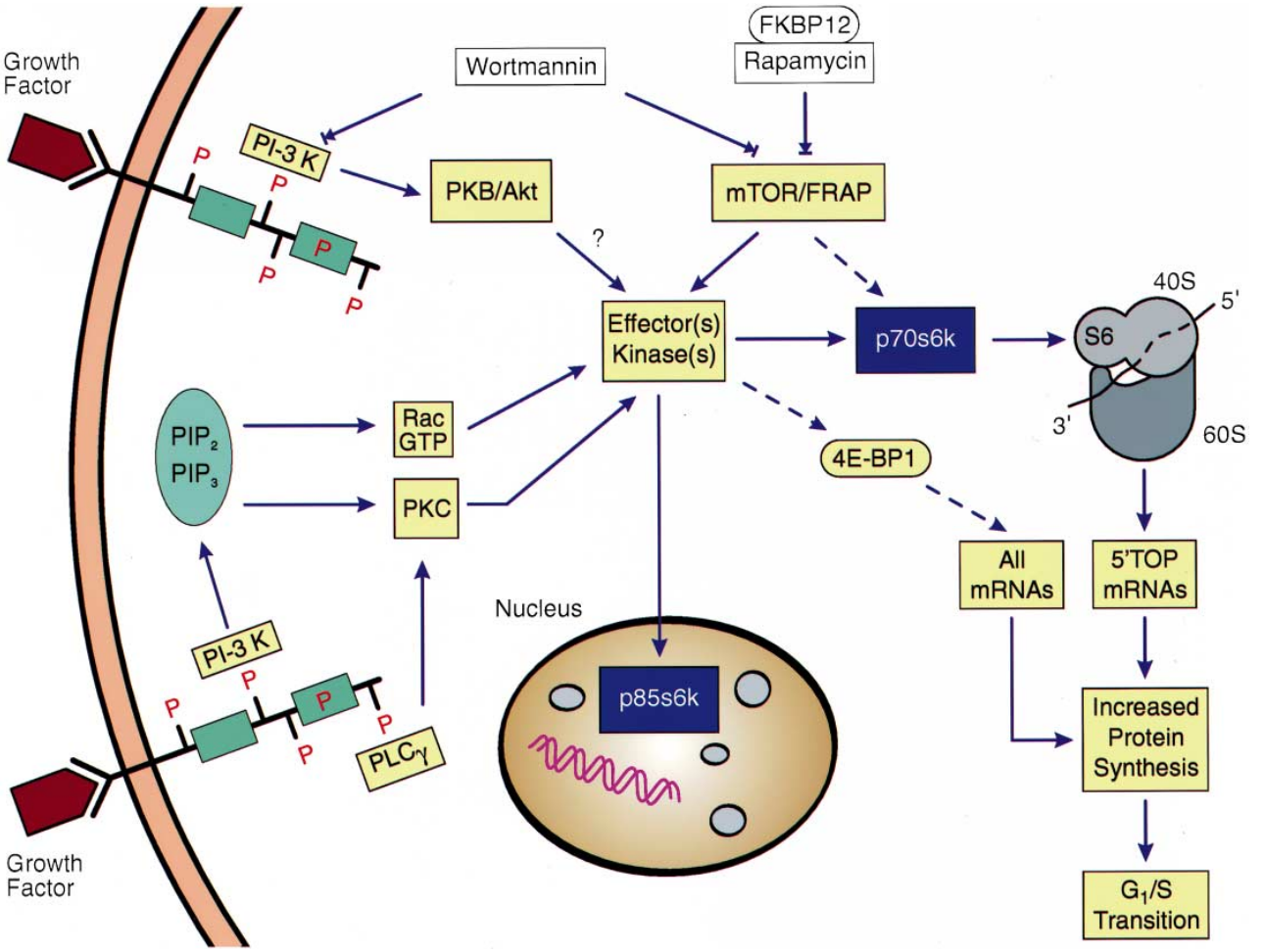

Fig. 1. The putative effectors of p70^{s6k} activity. Though the identification of the immediate upstream activators of p70^{s6k} is still awaited, this scheme highlights the potential candidates, their effectors and, in certain cases their inhibitors referred to in this review, whose activities are speculated to culminate in the activation of p70^{s6k}.

establish the mechanism by which mTOR/FRAP activity is modulated in response to mitogens and whether dominant-negative alleles of mTOR/FRAP actually interfere with the p70^{s6k} input.

Other pharmacological approaches have strongly indicated that a pathway leading to the activation of p70^{s6k} is initiated by the recruitment of the phosphatidylinositide-3-kinase (PI-3K) to the activated receptor [34,35]. This enzyme phosphorylates the D-3 position of PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 to produce second messengers which are thought to target a range of effector molecules [36], including the Rho family of G proteins [37], members of the novel and atypical PKCs [38,39] as well as the Ser/Thr kinase PKB/Akt [40–43]. Where mTOR/FRAP is located in this scheme is unclear (Fig. 1 and below). Constitutively active PI-3K alleles cause elevations in basal p70^{s6k} activity [35,42], whereas wortmannin and the structurally unrelated LY294002 compound inhibit PI-3K as well as the activation of p70^{s6k} in vivo [23,26,34,35,44]. However, not all the data from studies employing either receptor mutants [21] or dominant-negative molecules [45,46] are consistent with PI-3K activation being a necessary prerequisite for p70^{s6k} activation. Furthermore, the specificity of wortmannin and LY294002 for PI-3K have been recently challenged [31,44,47], thus pharmacology is unlikely to resolve this conflict and emphasises the need to identify the immediate effectors of p70^{s6k} activation.

PKB/Akt kinase, the insulin-responsive regulator of GSK-3 [48], is recruited to the membrane in response to increasing levels of PtdIns(3,4)P_2 [43]. Whilst PKB/Akt has not been shown to directly phosphorylate p70^{s6k}, over-expression of a *gag*-fusion of PKB/Akt constitutively activates p70^{s6k} [40]. More recently a PKB/Akt variant has been described which has acidic residues substituted at two key phosphorylation sites [49]. This mutant exhibits constitutive kinase activity and is resistant to wortmannin. If the wortmannin resistance of this mutant could be passed on to p70^{s6k} and a dominant-negative PKB/Akt could block p70^{s6k} activation, this would be compelling support for placing PKB/Akt as an upstream regulator of p70^{s6k}. TPA-induced activation of p70^{s6k} is also not significantly inhibited by wortmannin [23], but since the effects of TPA and constitutively active PKB/Akt can be blocked by rapamycin treatment, the implication is that PKB/Akt and PKC are either further upstream of mTOR/FRAP or reside on parallel pathways, signalling to a common component of the p70^{s6k} pathway.

Chou and Blenis [50] have shown that the small Rho family G proteins, rac-1 and cdc42, can bind in vitro to hypophosphorylated p70^{s6k} in a GTP-dependent fashion and GTPase-

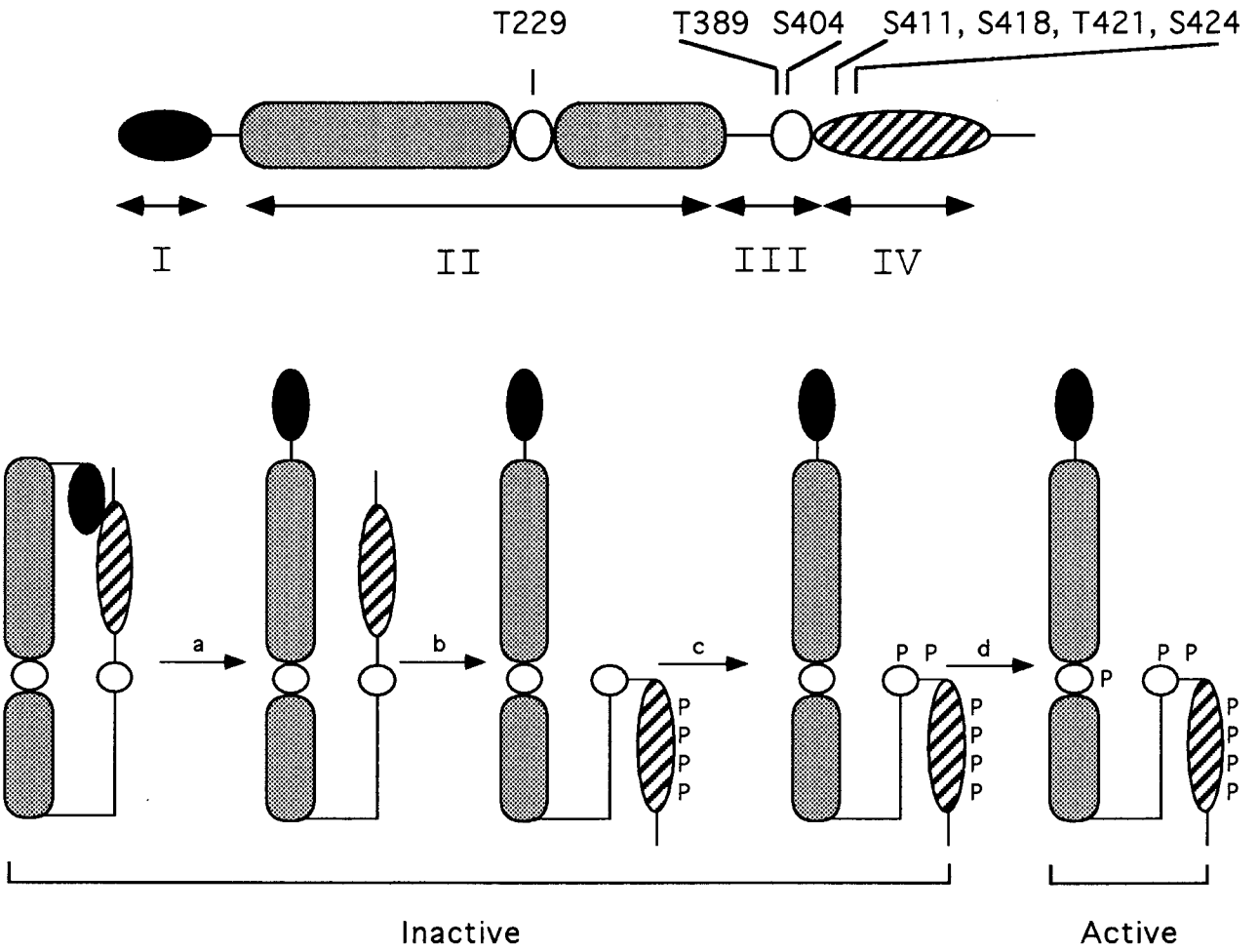

Fig. 2. (A) A modular representation of $p70^{s6k}$. The primary structure of $p70^{s6k}$ has been functionally dissected into four modules, indicated I–IV below the figure. Identified sites of phosphorylation sites are indicated. (B) The modular activation of $p70^{s6k}$. To generate $p70^{s6k}$ activity the critical sites in Module II, T229, and III, T389, must become phosphorylated. The four-step model presented here predicts that a functional interaction between Modules I and IV is broken and stabilised (a), by phosphorylation of the S/T-P sites in Module IV (b). This event, possibly mediated by mTOR/FRAP, would allow the mitogen-activated T389 kinase to phosphorylate its critical cognate site in Module III, thereby allowing the final T-loop phosphorylation at T229 (c), to propagate full kinase activity (d).

deficient alleles elevate $p70^{s6k}$ activity in vivo. These are potentially exciting observations, but it has yet to be resolved whether rac-1/cdc42 interact directly with $p70^{s6k}$ or if, instead, this event is dependent on a second molecule. The further troubling aspect of these studies is that the effect of activated alleles on $p70^{s6k}$ is blocked by wortmannin. This finding was rationalised by a model in which both rac-1 and PI-3K are required for $p70^{s6k}$ activation. The inference from this study is that, in the presence of active rac-1 alleles, basal levels of PI-3K are sufficient to activate $p70^{s6k}$. If this model is correct, then the ability of activated alleles of PI-3K, or PKB/Akt, to stimulate $p70^{s6k}$ should also be blocked by dominant-negative rac-1.

3. The modular phosphorylation and activation of $p70^{s6k}$

The most significant advance in our understanding of $p70^{s6k}$ regulation will most likely be realised from focusing efforts on the identification of the kinases which directly activate the enzyme. A knowledge of the specific changes in the phosphorylation status of $p70^{s6k}$ and the mechanism by which they bring about kinase activation is a necessary corollary to this.

In the absence of crystallographic data, structure/function relationships have been studied by the dissection of $p70^{s6k}$ primary structure into domains or modules (Fig. 2A). In brief, $p70^{s6k}$ can be roughly divided into four functionally significant modules. Module I extends from the N-terminus to the beginning of the catalytic domain and confers rapamycin sensitivity to $p70^{s6k}$ [24,32,33]. Module II contains the conserved catalytic domain, including the acute site of mitogen-induced phosphorylation in the activation T-loop [27,35]. Module III links the catalytic domain with the carboxyl tail, contains two additional sites of acute phosphorylation and is conserved in many members of the second messenger family of Ser/Thr kinases [27]. Finally, Module IV contains the putative autoinhibitory domain, which has significant homology with the substrate region of S6, and four closely clustered phosphorylation sites [22]. $p70^{s6k}$ activation by interactions of each of these modules has manifested itself as a hierarchy of interdependent phosphorylation events. Seven sites of phosphorylation have been identified in the endogenous enzyme (Fig. 2A), with a further five either confirmed or predicted through studies employing ectopically expressed kinase. The initially identified sites of phosphorylation, S411, S418, T421 and S424 are

all flanked by a proline in the +1 position and reside within the putative autoinhibitory domain of Module IV [22]. Peptides which correspond in sequence to this domain inhibit the kinase [51], consistent with the hypothesis that Module IV acts as an autoinhibitory domain. These four residues are hypophosphorylated in quiescent cells, and become hyperphosphorylated in response to serum [23]. Substitution of these S/T-P sites with alanines [23] suppresses activation of the kinase, whilst the corresponding acidic residue replacements raise basal kinase activity [10]. However, full kinase activation is accompanied by the acute phosphorylation of T229, in the T-loop, as well as T389 and S404 in Module III [23,27]. The distance between T389 and T229 is conserved in other kinases of the second messenger family that contain these homologous sites [27]. These residues are characteristically flanked by bulky aromatic residues; the sequence similarity extends throughout Module III, suggesting that this module may play a role as a general regulator of kinase activity in those kinases which contain these motifs.

Although T229 phosphorylation is critical in modulating kinase activity of $p70^{s6k}$ [27,35] and other mitogen-activated kinases, several lines of evidence indicate that T389 phosphorylation is the chief event through which full $p70^{s6k}$ activity is acceded. Firstly, although the inhibitory effects of rapamycin and wortmannin are exerted through the dephosphorylation of T229, T389 and S404 [23,27], the principal target is T389 [27,33]. The effects of these drugs on $p70^{s6k}$ can be suppressed by a T389E change, whereas a T389A substitution fully inactivates $p70^{s6k}$ [27]. Emerging data from this laboratory indicate that T389 phosphorylation modulates $p70^{s6k}$ activation through alterations in T229 phosphorylation (P.B. Dennis, N. Pullen and G. Thomas, unpublished). A T389E substitution elevates T229 phosphorylation, whereas T389A blocks it. T229 mutations to alanine or glutamic acid ablate kinase activity and do not potentiate T389 phosphorylation [27]. Thus a simple picture of $p70^{s6k}$ activation is one in which T389 phosphorylation regulates T229 phosphorylation in a coordinated and hierarchical fashion. It has been suggested that PI-3K may directly modulate the phosphorylation of T229 and $p70^{s6k}$ activation [35]. However, recent studies from this laboratory employing activated alleles of PI-3K and a construct lacking Modules I and II, $p70^{s6k}\Delta N_{54}\Delta C_{104}$, demonstrate that these effects, as previously shown with serum [33], are mediated by T389 phosphorylation (N. Pullen and G. Thomas, unpublished).

How then do these phosphorylation sites and modules interact with one another to bring about $p70^{s6k}$ activation? Recent studies from Avruch and collaborators [24] have demonstrated that removal of Module I severely impairs the ability of the kinase to be activated by mitogens. This deficiency also prevented mitogens from inducing T229 and T389 phosphorylation [33]. The effect of Module I truncation on activity can be rescued by removal of Module IV [24,32,33] or by simply replacing the S/T-P sites in this module with acidic residues (P.B. Dennis, N. Pullen and G. Thomas, unpublished). In both instances, the recovery in activity is accompanied by T229 and T389 phosphorylation [27,33]. Truncation of Module IV alone does not significantly effect basal kinase levels, whereas substitution of acidic residues in the S/T-P positions do [10,27]. Collectively, these data suggest Modules I and IV cooperate in signalling to T389 (Fig. 2B). The surprising observation that the $p70^{s6k}\Delta N_{54}\Delta C_{104}$ construct was resistant to the effects of rapamycin, whilst retaining wortmannin sensitivity [24,32,33], casts some doubt on the role of phosphorylation in governing $p70^{s6k}$ activation. In the absence of serum, this construct had high basal T229 phosphorylation [33], but low T389 phosphorylation and low kinase activity. Full kinase activity was accompanied by T389 phosphorylation, in response to serum, and the phosphorylation of T229 and T389 was fully protected in the presence of rapamycin [33]. This is in stark contrast to the behaviour of the wild type enzyme and made it difficult to rationalise mTOR/FRAP as the progenitor of either T389 or T229 kinase activity per se. Instead, if linked with $p70^{s6k}$ activation, mTOR/FRAP must mobilise the interaction between Modules I and IV for function. This further raises the significance of Module IV in modulating kinase activity by, firstly, regulating access to T389 and, secondly, by restricting the access to T229 until T389 is phosphorylated (Fig. 2B). We [33] and others [24,32] have speculated that removal of the N-terminus prevents an effector molecule from binding, an event which is possibly mediated by mTOR/FRAP and necessary for transducing the mitogenic signal through Module IV to T389 phosphorylation (Fig. 2B). In contrast to rapamycin, phosphorylation of T389 in the $p70^{s6k}\Delta N_{54}\Delta C_{104}$ mutant was completely abolished by wortmannin. Although kinase activity was ablated, under these conditions, T229 phosphorylation was unaffected [33]. Since full kinase activity, apparently, requires phosphorylation of T229 and T389, the identification of the kinases which mediate these phosphorylation events is clearly important. Nevertheless, the implications of these data are that T229 kinase activity is unregulated in vivo and only dependent on T389 phosphorylation (Fig. 2B), suggesting that unmasking upstream signalling elements will be best served by identifying the kinases which phosphorylate T389 and the S/T-P sites.

As described in Fig. 2B, the tentative model of $p70^{s6k}$ activation is presently thought to proceed by the binding of an effector molecule at the N-terminus which segregates it from the C-terminus. Hyperphosphorylation of the S/T-P sites in Module IV are thought to stabilise this interaction, allowing a mitogen-regulated kinase to phosphorylate T389 and, in turn, propagate phosphorylation of the T-loop site leading to full activity. With increased understanding of how each phosphorylation event can be transmitted to another, this should encourage workers to utilise this information in designing experiments which will ultimately assign names and faces to the kinases, phosphatases and effectors of the signalling pathway to $p70^{s6k}$.

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