Nucleic Acids Research, 1997, Vol. 25, No. 2 395–402

Cap-independent translation initiation in *Xenopus* oocytes

Brett D. Keiper and Robert E. Rhoads*

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, 1501 King's Highway, Shreveport, LA 71130-3932, USA

Received August 14, 1996; Revised and Accepted November 12, 1996

ABSTRACT

Eukaryotic cellular mRNAs contain a cap at their 5′-ends, but some viral and cellular mRNAs bypass the cap-dependent mechanism of translation initiation in favor of internal entry of ribosomes at specific RNA sequences. Cap-dependent initiation requires intact initiation factor eIF4G (formerly eIF-4γ, eIF-4Fγ or p220), whereas internal initiation can proceed with eIF4G cleaved by picornaviral 2A or L proteases. Injection of recombinant coxsackievirus B4 protease 2A into *Xenopus* oocytes led to complete cleavage of endogenous eIF4G, but protein synthesis decreased by only 35%. Co-injection of edeine reduced synthesis by >90%, indicating that eIF4G-independent synthesis involved ongoing initiation. The spectrum of endogenous proteins synthesized was very similar in the presence or absence of intact eIF4G. Translation of exogenous rabbit globin mRNA, by contrast, was drastically inhibited by eIF4G cleavage. The N-terminal cleavage product of eIF4G (cp_N), which binds eIF4E, was completely degraded within 6–12 h, while the C-terminal cleavage product (cp_C), which binds to eIF3 and eIF4A, was more stable over the same period. Thus, translation initiation of most endogenous mRNAs in *Xenopus* oocytes requires no eIF4G, or perhaps only cp_C, suggesting a cap-independent mechanism.

INTRODUCTION

The translation of most cellular mRNAs is thought to be initiated by a 5′-end-dependent mechanism involving protein synthesis initiation factors of the eIF4 group. These factors collectively bind the 7-methylguanosine-containing cap, unwind secondary structure in the mRNA and catalyze its binding to the 43S initiation complex to form the 48S initiation complex (1,2). Members of this group include eIF4E, a cap binding protein, eIF4A, an RNA helicase, eIF4B, which stimulates the helicase and has RNA annealing activity, and eIF4G (formerly eIF-4γ, eIF-4Fγ or p220), which acts as a linker in this process, specifically complexing with eIF4E, eIF4A and eIF3 to bring together the mRNA 5′-end, the RNA helicase activity and the 40S subunit (3). The isolated complex of eIF4E, eIF4A and eIF4G is referred to as eIF4F.

In some situations, however, cap-independent initiation becomes predominant. The best understood instance of a switch from cap-dependent to cap-independent initiation involves picornavirus infection of mammalian cells. Picornaviral RNA contains an internal ribosome entry site (IRES) which is capable of productively binding ribosomal subunits and initiating translation independent of the 5′-end, even directing translation initiation on circular RNA (reviewed in 4). Viral mRNAs are not alone in their use of IRES elements as a means to utilize a cap-independent mechanism for initiation. mRNAs encoding the cellular proteins immunoglobulin heavy chain binding protein, fibroblast growth factor 2, *Drosophila* Antennapedia and yeast TFIID and HAP4 also contain IRESes and can initiate translation internally (4,5). Even the mRNA for initiation factor eIF4G itself has been found to contain a potent IRES (6). Picornaviral infection results in a dramatic shutdown of host cell protein synthesis (7). Within a few hours following infection by poliovirus, synthesis of cellular proteins is nearly undetectable while synthesis of viral polyproteins rapidly becomes predominant. Entero- and rhinoviruses contain a protease (protease 2A) which cleaves eIF4G at a specific site (amino acid 486 of rabbit eIF4G; 8,9), physically separating the domains of eIF4G which bind eIF4E from those which bind eIF4A and eIF3 (3,10) and resulting in the separation of the cap binding function from the RNA-unwinding and ribosome-binding functions of eIF4G. Under these conditions, cap-dependent initiation is inhibited and cap-independent initiation prevails in the cell. Cleavage of eIF4G by protease 2A correlates with the shutdown of host protein synthesis and is thought to be an important step in viral take-over of the protein synthetic apparatus of the host cell. Translation of cellular mRNAs and mRNA cap-binding activity can be restored to virus-infected cell extracts by adding the intact eIF4F complex (11,12). eIF4 factors, therefore, are at the center of the mechanistic decision to switch from cap-dependent to cap-independent initiation.

The relative contributions of cap-dependent and cap-independent initiation *in vivo* can be conveniently addressed in *Xenopus* oocytes. Fully grown oocytes are arrested both in cell cycle and development, yet synthesize protein actively (13,14). The translational efficiency of *Xenopus* stage VI oocytes is comparable with that of mammalian reticulocytes at similar temperatures (22°C; 15). They have amassed stores of maternally inherited mRNAs which provide genetic information for subsequent embryonic development. Following transcription and splicing, many of these mRNAs are transported from the nucleus in a

* To whom correspondence should be addressed. Tel: +1 318 675 5161; Fax: +1 318 675 5180; Email: rrhoad@lsumc.edu

ribonucleoprotein complex (mRNP), masked from the translational machinery in the cytoplasm by RNA-binding proteins which include FRGY2, and do not enter the pool of mRNA available for translation (16–18). Despite this fact, the pool of available mRNA remains in excess of the translation initiation potential of the non-stimulated oocyte. Studies utilizing microinjection of mRNAs have demonstrated that they compete with endogenous mRNAs and with each other for the existing initiation machinery (19–21). The limiting activity is thought to be that of a member of the eIF4 group of initiation factors (22). The stored maternal mRNAs remain untranslated until recruited for translation in response to a developmental stimulus such as progesterone or fertilization (reviewed in 23,24). Their utilization in a cell cycle-dependent manner is likely mediated both by unmasking of these mRNAs and regulation of the translation initiation apparatus (25).

In this study we address cap-dependent and cap-independent initiation in *Xenopus* oocytes by microinjecting purified recombinant protease 2A from coxsackievirus B4 (CVB4) to bring about proteolytic cleavage of eIF4G. Since oocytes are both synthetically active and non-proliferating, we have been able to focus on the isolated effects of disrupting eIF4G function on overall translation in a single, living cell. We find that eIF4G cleavage severely inhibits cap-dependent initiation but causes only minor inhibition of total protein synthesis. The results suggest that much of the actively translating mRNA in the oocyte may initiate translation via a cap-independent pathway, although there is no evidence at present to suggest this is internal initiation.

MATERIALS AND METHODS

Picornaviral protease 2A

Recombinant coxsackievirus B4 protease 2A (CV2A) was a gift of Dr Barry Lamphear (Louisiana State University Medical Center). It was produced in an overexpressing *Escherichia coli* strain (a gift of Dr Tim Skern, University of Vienna, Vienna, Austria) and purified to homogeneity as previously described (26,27). Protease was diluted at least 10-fold in modified Barth's saline solution (MBS; 28) just prior to microinjection and kept on ice.

Xenopus oocyte isolation, microinjection and culture

Frogs were purchased from Xenopus I (Madison, WI). Ovaries were surgically removed from non-hormone-stimulated females and rinsed with MBS. Oocytes were isolated manually with watchmakers forceps and a toothpick, sorted to select stage VI oocytes and remove those with blemishes, and cultured at room temperature (22–24°C) in MBS containing 10 µg/ml penicillin G and 10 µg/ml streptomycin sulfate. Solution volumes of 20, 25 or 30 nl were microinjected equatorially into the oocyte cytoplasm using a Nanoject (Drummond, King of Prussia, PA) delivery system and beveled tip (30 µm) microcapillary needles (28). The follicle cells surrounding individual oocytes were *not removed* by collagenase/pronase treatment due to the dramatic adverse effects on protein synthesis in the 24 h period following treatment (29). Rabbit globin mRNA was isolated as described (30) and then further purified by a second round of oligo(dT)–cellulose chromatography (31).

Metabolic labeling of oocyte protein synthesis *in vivo*

Metabolic labeling was used in preference to microinjection of radioactivity to prevent distortion of the endogenous amino acid pool, to allow more precise dosage to all of the experimental oocytes and to provide a continuous supply of radiolabel over the course of several hours which was not subject to depletion or leakage. After culturing, groups of three oocytes (in either duplicate or triplicate) were transferred to 1.5 ml microcentrifuge tubes and excess buffer removed with a drawn Pasteur pipette. Labeling was begun by the addition of 20 µl 0.5 mCi/ml [^{35}S]methionine (>1000 Ci/mmol; ICN Radiochemicals) in MBS at room temperature (22–24°C). Labeling was ended by rinsing oocytes with 0.5–1 ml MBS twice and freezing in dry ice. Oocytes were homogenized at 4°C in extraction buffer (EB; 50 mM Tris–HCl, pH 7.5, 0.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride) with microtube pestles (Sarstedt), centrifuged at 25 000 *g* for 5 min in a microfuge and aliquots from the supernatant spotted to duplicate Whatmann 540 filters. One filter from each sample was washed in 5% (w/v) trichloroacetic acid (TCA), 1 mM methionine for ≥30 min, boiled in 10% (w/v) TCA for 5 min, rinsed in methanol, rinsed in acetone, air-dried and the radioactivity measured by liquid scintillation spectrometry. The second filter was air-dried directly for assay of radioactivity by liquid scintillation spectrometry and served as a measure of total radioactive uptake by the oocytes. The initial rate of [^{35}S]methionine uptake by oocytes was always much greater than the rate of incorporation into TCA-precipitable material and was essentially unaffected by the various treatments, indicating that the rate of incorporation represented the rate of protein synthesis. Nevertheless, equilibration with the oocyte methionine pool caused an ~10 min lag in incorporation into protein (data not shown) and may have caused a slight underestimation of protein synthesis during the initial 20 min of labeling. After 20 min, complete equilibration had occurred, as demonstrated by the parity of radioactivity between extract and labeling medium. Under these conditions, oocytes generally demonstrated linear incorporation of radioactivity into protein following the lag period for at least 3 h. Estimates of the endogenous methionine pool (43 pmol/oocyte; 32) and nominal oocyte volume (1 µl; 28) were used to calculate synthetic rates.

Preparation of total oocyte extracts, electrophoresis and Western analysis

Oocytes were homogenized at 4°C in buffer EB, centrifuged at 25 000 *g* for 5 min and the supernatants resolved by SDS–PAGE. Immunoblotting for eIF4G was performed using N-terminal antiserum which recognizes amino acids 327–342 (33) or C-terminal antiserum which recognizes amino acids 653–666 (3). Radiolabeled extracts were resolved by SDS–PAGE on 10 or 15% gels, which were subsequently fixed in 45% methanol, 5% acetic acid then soaked in 1 M sodium salicylate prior to drying and autoradiography. Image analysis was by PhosphorImager detection with quantitation using ImageQuant (Molecular Dynamics) or by scanning on a Hewlett Packard ScanJet with quantitation by NIH Image v1.5 software.

RNA analysis

Total RNA was prepared from oocytes by the SDS/proteinase K method (34). RNA from two oocytes was resolved by

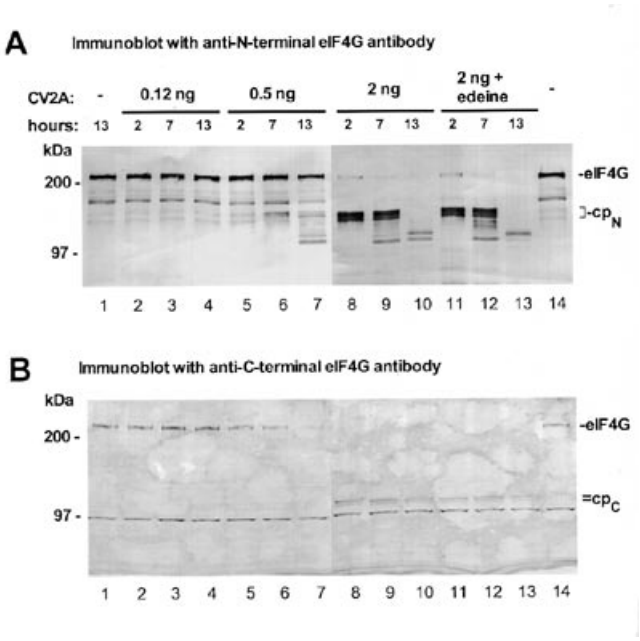

Figure 1. Cleavage of *Xenopus* eIF4G by microinjected CV2A. Oocyte extracts were analyzed by Western blotting with antisera against the N-terminal (**A**) and C-terminal (**B**) regions of human eIF4G. Oocytes were injected with buffer (lane 1), 0.12 (lanes 2–4), 0.5 (lanes 5–7) or 2 ng CV2A (lanes 8–10) or 2 ng CV2A plus 500 µM edeine (lanes 11–13). Oocytes were incubated at 20°C for 2 h (lanes 2, 5, 8 and 11), 7 h (lanes 3, 6, 9 and 12) or 13 h (lanes 1, 4, 7, 10 and 13) prior to freezing in groups of three. Extract equivalent to 0.25 oocytes was resolved by electrophoresis and immunoblotted. The positions of intact eIF4G, the heterogeneous N-terminal cleavage product (cp_N) and a doublet of 100 kDa corresponding to the C-terminus of eIF4G (cp_C) are indicated.

formaldehyde–1.5% agarose gel electrophoresis and capillary blotted to NitroPlus 2000 membranes (MSI Separations) (35). An antisense RNA probe to endogenous B9 mRNA (36) was transcribed with T7 RNA polymerase (Promega) and [α-^{32}P]UTP (Dupont/NEN) from linearized plasmid. An antisense DNA globin probe was primer extended on plasmid pOG9 (37), encoding a rabbit β globin cDNA, using [α-^{32}P]dCTP (Dupont/NEN) (35).

RESULTS

To verify that antibodies against human eIF4G would specifically recognize eIF4G from *Xenopus* oocytes, we utilized anti-peptide antibodies directed at N- and C-terminal portions of human eIF4G protein (3,33) for Western blotting of extracts of whole oocytes. A closely spaced triplet of polypeptides migrating on SDS–PAGE with an apparent molecular mass of ~230 kDa reacted strongly with the N-terminal antibody (Fig. 1A). Mammalian eIF4G is also heterogeneous on SDS–PAGE, possibly due to post-translational modification (3). The other, fainter bands at ~150 kDa on the Western blot may represent an N-terminal breakdown product of eIF4G or a full-length eIF4G polypeptide which has not undergone the putative post-translational modification. The C-terminal antibody also recognized the ~230 kDa proteins, though with reduced sensitivity (Fig. 1B).

The susceptibility of *Xenopus* eIF4G to CV2A was tested. Coxsackievirus B4 grows in a human host and, to date, CV2A has been shown to cleave eIF4G from only human and rabbit sources (8,27). Microinjection of recombinant CV2A into stage VI *Xenopus* oocytes resulted in specific cleavage of the endogenous eIF4G in a dose- and time-dependent manner (Fig. 1). This protease exhibits a high degree of substrate site specificity, recognizing peptides bearing the sequence Ile/Leu-X-Thr-X*Gly-Pro and cleaving the X*Gly peptide bond (38). The introduction of as little as 0.5 ng CV2A (25 nM final concentration) caused detectable cleavage of eIF4G in a 7 h incubation, as evidenced by the appearance of a set of N-terminal cleavage products, cp_N, which migrated with apparent molecular masses of 130–140 kDa (Fig. 1A, lane 6). Immunoreactive bands at ~100 and ~110 kDa (lanes 7, 9, 10, 12 and 13) are likely to arise from secondary cleavage of cp_N by cellular proteases, since they do not appear when eIF4G is initially cleaved to cp_N but rather appear coincident with cp_N loss. *Xenopus* eIF4G was cleaved to completion in 7 h by 2 ng CV2A (100 nM final concentration). The residual eIF4G signal derives from the thin layer of follicle cells surrounding the oocyte, as was demonstrated by Western blotting of extracts from CV2A-treated oocytes denuded of the follicle cells either manually or by treatment with collagenase (data not shown). cp_N accumulated only transiently, presumably being degraded by cellular proteases (lanes 10 and 13). The same oocyte extracts were probed for the presence of cp_C, the C-terminal portion of eIF4G. cp_C appeared as a doublet of 100 kDa (Fig. 1B, lanes 8–13), which is similar in size to human cp_C (102 kDa; 8). In contrast to cp_N, *Xenopus* cp_C was considerably more stable in the oocyte over the 11 h following cleavage of eIF4G (compare Fig. 1A and B, lanes 8–10). Thus, if any residual role in initiation is played by a fragment of the eIF4G molecule, it must be by cp_C.

The effect of eIF4G cleavage on the protein synthetic capacity of oocytes was addressed by metabolically labeling with [^{35}S]methionine (Fig. 2). A higher range of CV2A doses was used to cleave eIF4G either partially (0.4 ng) or completely (2 and 10 ng) in a 12 h period. Oocytes were subsequently pulse-labeled and protein extracts from duplicate labelings resolved by SDS–PAGE. Injection of 10 ng CV2A resulted in cleavage of essentially all of the eIF4G detected by Western blotting within 2 h (Fig. 2A). Surprisingly, the spectrum of proteins synthesized by these oocytes 10 h later was not remarkably different from sibling oocytes in which only 10% of the eIF4G was cleaved nor from those injected with buffer alone (Fig. 2B). While most protein bands remained the same, a few disappeared, while several others were enhanced by cleavage of eIF4G. The latter included the most abundant protein resolved in these gels, cytoskeletal actin (p45, whose identity was verified by immunoprecipitation; data not shown). Actin synthesis increased in all experiments (n = 6), although the extent of increase varied. Changes in the types of proteins synthesized correlated with more extensive cleavage of eIF4G. The synthesis of actin, for example, was stimulated ~2-fold in this experiment, but only in oocytes where eIF4G cleavage was complete (Fig. 2B, lanes 5–8).

We addressed the nature of the eIF4G-independent incorporation of [^{35}S]methionine by using specific inhibitors of protein synthesis. To verify that the incorporation of radioactivity after eIF4G cleavage was due to protein synthesis, we preincubated oocytes in 35 µM cycloheximide or injected them with 10 ng CV2A, which was more than sufficient to cleave all of the oocyte eIF4G within 2 h, and then labeled oocytes with [^{35}S]methionine for various times (Fig. 3A). Cycloheximide completely abolished incorporation of radioactivity, indicating that the [^{35}S]methionine incorporation by eIF4G-deficient oocytes was due exclusively to

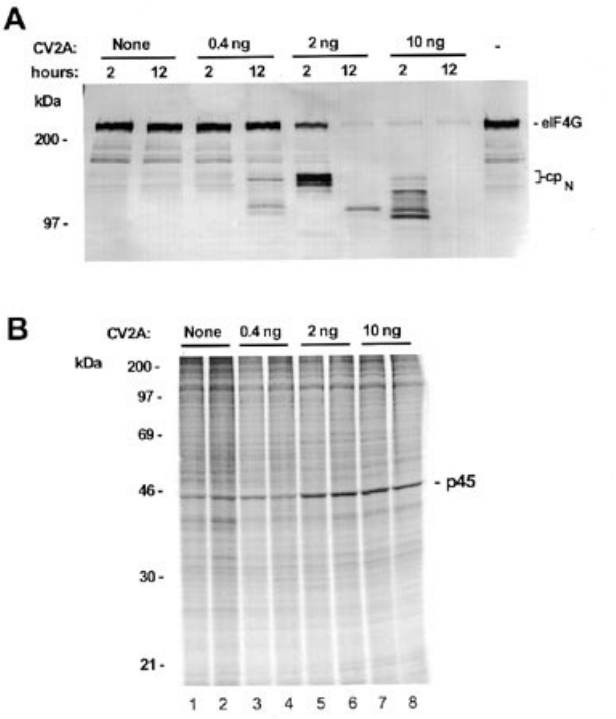

Figure 2. Relationship of protein synthesis to eIF4G cleavage. (**A**) Western blotting of oocyte extracts with N-terminal antiserum against eIF4G. Oocytes were injected with the indicated amounts of CV2A and incubated at 20°C for 2 or 12 h prior to freezing. Samples were prepared and analyzed as in Figure 1A. (**B**) Sibling oocytes were incubated for 12 h and then metabolically labeled for 1 h with [^{35}S]methionine. Extracts from duplicate groups of oocytes (lanes 1 and 2, 3 and 4, 5 and 6 and 7 and 8) were analyzed by SDS–PAGE. p45 indicates the position of cytoplasmic actin.

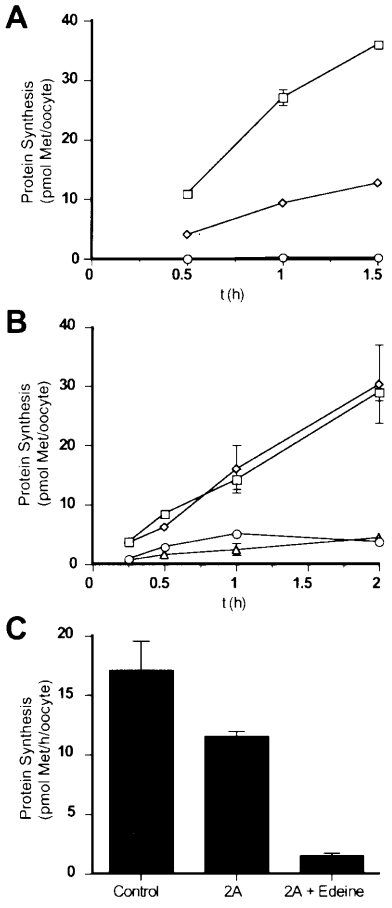

Figure 3. eIF4G-independent translation initiation. Total protein synthesis was assayed from duplicate groups of oocytes by metabolic labeling with [^{35}S]methionine. (**A**) Oocytes were injected with 10 ng CV2A protease (◇) or buffer (□) and incubated for 2 h to allow complete cleavage of eIF4G (data not shown) prior to labeling. Oocytes were incubated in 35 µM cycloheximide (○) for 2 h prior to and during labeling to abolish synthesis. (**B**) Edeine was injected to final concentrations of 0 (□), 1 (◇), 10 (○) and 100 (Δ) µM and oocytes incubated for 2 h prior to metabolic labeling with [^{35}S]methionine. Linear regression of these curves indicated that 10 µM edeine inhibited 90.2% of total synthesis. (**C**) Synthesis during a 1 h labeling following injection of 2 ng CV2A (2A) or co-injection of 2 ng CV2A and 500 µM edeine (2A+Edeine; 10 µM final edeine concentration).

protein synthesis. [^{35}S]Methionine incorporation in CV2A-treated oocytes was nearly linear for 1.5 h at a rate 34% that of buffer-injected oocytes. The 10 ng dose of CV2A was in excess of that required for complete eIF4G cleavage in these experiments and tended to produce toxic effects upon extended incubation which were evident in oocyte morphology. Hence, a lower rate of protein synthesis was observed here than in subsequent experiments using 2 ng CV2A.

To test whether the residual incorporation of [^{35}S]methionine after eIF4G cleavage was due solely to translation elongation, we used the initiation inhibitor edeine. Edeine blocks joining of 60S subunits following 48S complex formation on mRNAs and has been used extensively to demonstrate 'scanning' by the 40S subunit (39,40). Labeling of oocytes following injection of various edeine doses indicated that 10 µM was sufficient to produce maximal inhibition of protein synthesis (Fig. 3B). Edeine at 10 µM inhibited the rate of protein synthesis by 90%, indicating that most of the protein synthesis assayed in the 2 h labeling period was due to *de novo* initiation events. It was then possible to measure the proportion of eIF4G-independent synthesis which was due to initiation rather than elongation by combining the inhibitory effects of CV2A and edeine. Oocytes lacking intact eIF4G synthesized protein at a rate of 11.6 ± 0.5 pmol methionine/h/oocyte, or 68% of control cells (Fig. 3C). Co-injection of edeine reduced the rate of synthesis to 1.6 ± 0.3 pmol methionine/h/oocyte. The difference between these measurements (10.0 ± 0.8 pmol methionine/h/oocyte, 86% of the rate without edeine) represents *de novo* initiation which occurs without intact eIF4G. Thus, most of the protein synthesis observed in a 1 h pulse labeling of eIF4G-deficient oocytes was due to *de novo* initiation.

The finding that the major portion of protein synthesis is not sensitive to eIF4G cleavage suggests that either the cap-dependent initiation pathway is not operative in stage VI oocytes or that only a small subset of mRNAs utilize the cap-dependent pathway, while the majority utilize the cap-independent pathway. To confirm that the cap-dependent pathway was still operative, we injected purified rabbit globin mRNA into oocytes in which eIF4G was cleaved. The cap dependence of rabbit globin mRNA has been well documented (41) and its utilization in *Xenopus* oocytes has also been described in detail (13,20,21). Oocytes were injected with globin mRNA either before or after injection of CV2A protease (Fig. 4). In the former case, globin mRNA had ample opportunity (13 h) to form polyribosomes before eIF4G

cleavage (19); in the latter, eIF4G was cleaved before the mRNA was introduced. Seven hours after CV2A injection, oocytes were pulse-labeled with [^{35}S]methionine for 1 h. eIF4G cleavage was complete or nearly complete by the end of the labeling period based on Western blotting of the labeled extracts (Fig. 4A). At a dose of 5 ng, globin mRNA directed synthesis of the most highly labeled protein resolved in the low molecular weight range by SDS–PAGE and began to compete with endogenous mRNA for translation (Fig. 4B, lanes –, gb/bf, bf/gb and –/gb). Densitometry scanning of the gel in Figure 4B indicates that, in globin-injected oocytes lacking CV2A, endogenous protein synthesis was reduced to 73 ± 15% of that in non-injected control cells. The rate of globin synthesis was constant in buffer-injected cells (Fig. 4B, lanes gb/bf and bf/gb), despite the fact that globin mRNA was introduced at very different times prior to the labeling (19 or 4 h). This indicates efficient reinitiation of the mRNA and the absence of significant mRNA degradation. However, when eIF4G was cleaved in these oocytes, globin translation was completely abolished (Fig. 4B, lanes gb/2A and 2A/gb). The synthesis of globin in the absence of CV2A treatment was 4800-fold and 13 000-fold greater, respectively, than that after eIF4G was cleaved. The lack of globin synthesis was not due to degradation of globin mRNA, as demonstrated by Northern blot analysis at the time of labeling (Fig. 4C). As observed in Figure 2, translation of endogenous mRNAs was only moderately inhibited and the pattern of endogenous protein mostly unchanged by CV2A injection. These results demonstrate that the cap-dependent pathway is operative in stage VI oocytes and that it is exceedingly sensitive to eIF4G cleavage. Furthermore, the sensitivity of cap-dependent translation to eIF4G cleavage is observed regardless of whether the test mRNA (globin) is preloaded on polyribosomes or introduced after eIF4G cleavage.

To obtain a more quantitative measure of eIF4G-independent initiation in oocytes, we injected 1 ng CV2A into oocytes previously loaded with 5 ng globin mRNA. Under these conditions, cleavage of eIF4G proceeded gradually to completion over a period of 9 h (Fig. 5A). During this time, 1 h pulse labelings were carried out and globin synthesis as well as endogenous protein synthesis were measured. The total protein synthetic rate decreased by 35% after 3–4 h, a time when 20% of the eIF4G remained intact (Fig. 5B). The rate of synthesis stabilized at 65–70% of maximal despite a further reduction in eIF4G over the next 5 h, suggesting that all of the eIF4G taking part in protein synthesis had been cleaved. These results are also in good agreement with our estimate of the proportion of eIF4G-independent synthesis from Figure 3. The rate of globin synthesis in these oocytes, however, differed markedly from the synthesis of most endogenous proteins. Globin synthesis dropped to <10% of control within 3–4 h, correlating closely with the extent of eIF4G cleavage (Fig. 5B) and indicating a very strong dependence on intact eIF4G and the cap-dependent pathway. These data show that while only 35% of total endogenous protein synthesis is dependent on intact eIF4G in *Xenopus* oocytes, >90% of the synthesis of a highly cap-dependent mRNA depends upon intact eIF4G.

DISCUSSION

Most picornaviruses target eIF4G for inactivation as a means of disrupting cap-dependent translation initiation in order to favor the translation of their genomic RNAs, which do not contain caps (reviewed in 4). These viruses encode a specific protease, 2A,

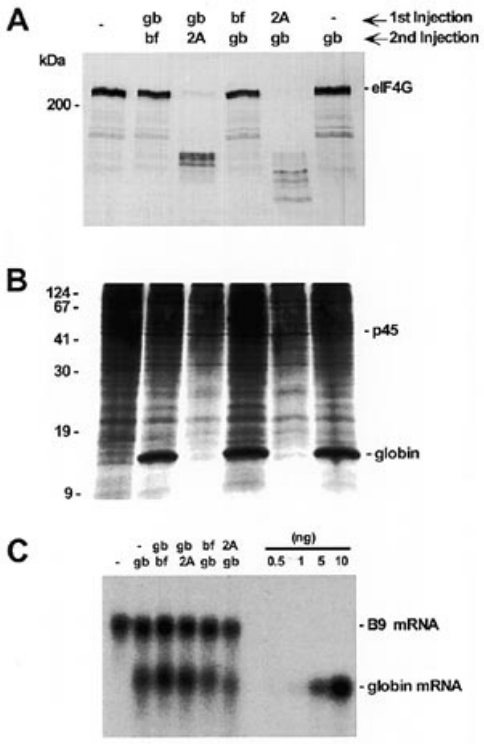

Figure 4. Inhibition of globin mRNA translation by eIF4G cleavage. Oocytes were injected with 5 ng purified rabbit globin mRNA (gb) either 13 h prior to or 2 h after injection of 2 ng CV2A protease (2A) or buffer (bf). – refers to oocytes which were not injected. Seven hours after CV2A injection, all oocytes were metabolically labeled with [^{35}S]methionine for 1 h. (**A**) Western blot analysis of ^{35}S-labeled extracts with the N-terminal antibody at the end of the labeling period. (**B**) Autoradiography of the extracts in (A) resolved by SDS–PAGE. The positions of migration of globin and *Xenopus* actin (p45) are shown. Inhibition of total protein synthesis in gb/2A and 2A/gb was 51 and 62% respectively. The synthesis of endogenous actin (p45) relative to total synthesis was stimulated 1.7- and 2.0-fold in this experiment. (**C**) Northern blot analysis of sibling oocytes frozen at the time of ^{35}S labeling. The positions of *Xenopus* maternal mRNA B9 and injected rabbit β-globin are shown. Lane designations are as used in (A) and (B). Lanes 7–10 were loaded with 0.5, 1, 5 and 10 ng purified rabbit globin RNA as standards.

which cleaves the eIF4G molecule (8,38), effectively separating the domain which binds to the mRNA cap-binding protein, eIF4E, from that which interacts with the rest of the 48S initiation complex (3,10). Cleavage of eIF4G in mammalian cells infected with poliovirus, for example, correlates with a dramatic shutdown of host cell translation within 2–3 h of infection (9). Intact eIF4F complex purified from non-infected cells restores active translation and mRNA cap-binding function to extracts derived from poliovirus-infected cells, indicating that the only lesion to protein synthesis is the inactivation of this cap-binding complex by 2A cleavage (11,12). Mutations in the viral 2A gene which inactivate the protease alleviate the shutdown of host cell synthesis during infection (42,43), again linking eIF4G cleavage to the loss of cellular mRNA translation. Thus, there is much correlative evidence from studies of picornaviral infection that the cleavage of eIF4G by protease 2A leads to host synthesis shutdown.

However, there is also evidence to the contrary, i.e., that the cleavage of eIF4G cannot be solely responsible for the dramatic shutdown of host protein synthesis. Temporally, host protein

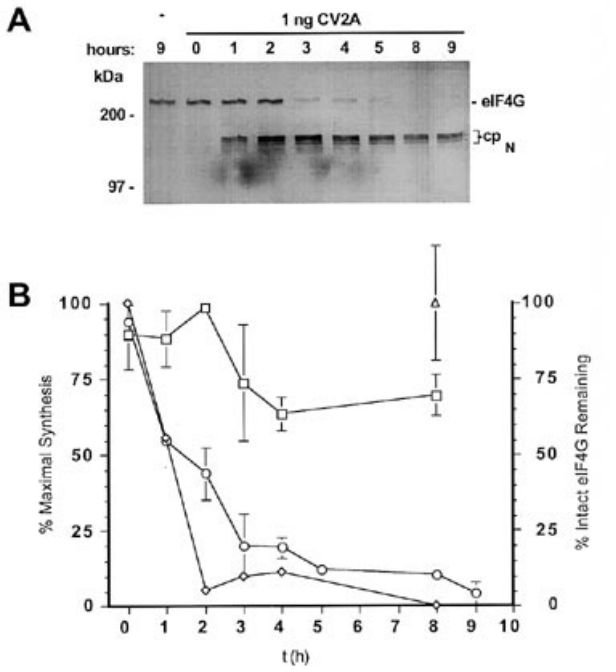

Figure 5. Extent of cap-independent translation initiation in *Xenopus* oocytes. Oocytes were preloaded with 5 ng globin mRNA 12 h prior to the beginning of the time course. The injection of 1 ng CV2A was defined as $t = 0$. Triplicate groups of oocytes were removed at various times and metabolically labeled with [^{35}S]methionine for 1 h. (**A**) Western blot analysis of oocytes frozen at 0, 1, 2, 3, 4, 5, 8 and 9 h after injection of CV2A. (**B**) Cleavage of *Xenopus* oocyte eIF4G (○) and total protein synthesis (□) and rabbit globin synthesis (◇) over the 9 h time course. The extent of eIF4G cleavage was calculated from (A) and a similar blot and expressed as percentage intact eIF4G relative to the sum of intact and cp_N species. Values for total protein and globin synthesis are plotted at the beginning of 1 h pulse labeling periods and are expressed as a percentage of control oocytes (Δ). Globin synthesis was quantitated by SDS–PAGE and Phosphorimager analysis of a single gel.

synthesis shutdown actually lags behind eIF4G cleavage by ~1 h in poliovirus-infected cells; this lag can be extended to at least 5 h when infected cells are shifted to 28°C (44). Prior to the shutdown of cellular synthesis, IRES-directed translation has already been stimulated, indicating that separate mechanisms mediate these events (45). In the presence of certain ionophores and agents which inhibit replication of the viral genome, complete eIF4G cleavage still occurs, but cellular protein synthesis continues at 50–100% of the rate of uninfected cells, leading to simultaneous translation of cellular and viral RNAs (46,47). These seemingly contradictory observations may possibly be reconciled if one considers that virus infection produces changes in intracellular cation concentrations, membrane permeability and cytoskeletal structures, which could also inhibit protein synthesis (48–50). Picornavirus infection also leads to increased phosphorylation of eIF2α by PKR in reponse to dsRNA and interferon, causing inhibition at another step of translation initiation (7).

Simplified systems in which protease 2A alone is added may provide more insight into the effects of eIF4G cleavage on protein synthesis. Translation of globin mRNA is dramatically inhibited by eIF4G cleavage *in vitro* using reticulocyte lysate treated with purified picornavirus protease 2A (26,51), similar to our observations in oocytes. Likewise, the action of FMDV leader protease inhibits the translation of capped mRNAs to various extents (52,53). On the other hand, eIF4G cleavage can lead to stimulation of cap-independent initiation. Translation of RNAs containing some picornaviral IRESs is stimulated 6- to 10-fold in lysates treated with protease 2A (51,54). Translation of several cellular mRNAs introduced as uncapped RNAs is also stimulated 2- to 5-fold by eIF4G cleavage (53). The simple interpretation from the *in vitro* studies is that cleavage of eIF4G alone is sufficient both to disrupt cap-dependent translation and facilitate cap-independent translation, be it IRES-mediated or non-IRES-mediated.

Because the stable expression of protease 2A in cells appears to be quite toxic (55), analyzing the specific effects of eIF4G cleavage on translation *in vivo* has been more complicated. To date, transient transfection of mammalian cells with 2A-expressing vectors has been the only means to approach these questions *in vivo*, but it is clear from these studies that eIF4G cleavage leads to profound translational inhibition of specific cap-dependent mRNAs (56,57). The stimulation of IRES-mediated translation initiation as well as that of uncapped cellular mRNAs has also been borne out by these transfection studies (45,57), again indicating that eIF4G cleavage inhibits the cap-dependent pathway and stimulates the cap-independent pathway. However, the contribution of eIF4G cleavage to host protein synthesis shutdown has not been addressed in these studies, as only a subset of cells express protease 2A. As for the toxic effects of 2A expression, they are difficult to assess in growing somatic cells, where the secondary effects of inhibited translation of a specific mRNA, for example, may impair the cell's subsequent ability to carry out DNA replication, transcription or cell cycle events. In the present study, we have employed a new *in vivo* system to assess the role of eIF4G cleavage in cap-dependent and cap-independent translation by injecting CV2A into quiescent *Xenopus* oocytes.

It is clear that the cap-dependent initiation pathway is active in oocytes. Indeed, we observe that several species of ^{35}S-labeled proteins disappear upon cleavage of eIF4G, though they are surprisingly few. A clearer demonstration, though, is that translation of globin mRNA is completely abolished by cleavage of eIF4G. Globin mRNA is efficiently utilized in stage VI oocytes, forming polyribosomes within 3–4 h of injection and completing a round of translation every 5–10 min; its utilization is 10- to 30-fold more efficient than in reticulocyte lysate (13,21). Early studies found that globin mRNA competes in a dose-dependent manner in the range 1–100 ng with endogenous mRNAs (19–21). The authors of these studies concluded that protein synthesis in the stage VI oocyte is limited by a saturated translational capacity (e.g. factors, subunits), not by limiting mRNA availability. We carefully chose the 5 ng dose of globin mRNA in order to effect moderate competition such that globin translation would accurately reflect the cap-dependent utilization of the available mRNA pool. The complete inhibition of globin mRNA translation is evidence that the cap-dependent pathway was fully disrupted. Preloading of globin mRNA into polyribosomes before eIF4G cleavage did not prevent inhibition, suggesting that each new round of initiation involves a cap-dependent event. In general, the presence of a cap on an injected mRNA enhances its translation ~25-fold in oocytes, whereas it increases stability only 5-fold (58). Translation of exogenous globin mRNA provides a very sensitive assay of cap-dependent translation initiation activity and is highly dependent on the integrity of eIF4G.

In contrast to globin mRNA, most endogenous mRNAs in the stage VI oocyte can be translated in the absence of intact eIF4G, suggesting a cap-independent pathway. Unlike virus-infected mammalian cells, *Xenopus* oocytes do not undergo a dramatic shutdown of host cell protein synthesis when eIF4G is cleaved. Instead, only ~35% of cellular protein synthesis is inhibited. Given that translational efficiency and ribosome transit times measured in *Xenopus* stage VI oocytes were found to be comparable with mammalian systems (15), these results cannot be reconciled by slow elongation rates in oocytes. Nor is the modest inhibition by protease 2A due to low initiation activity in oocytes, since edeine injection reveals that at least 90% of synthesis measured within the 2 h labeling period is due to new initiation events. Edeine binds to the small ribosomal subunit (59,60) and prevents joining of the large subunit to a 48S initiation complex, probably by blocking initiation codon recognition by the scanning 40S subunit (39,40). Thus, edeine acts at an event subsequent to cap recognition and should inhibit both cap-dependent and cap-independent initiation events, but not elongation (39). By using edeine in conjunction with eIF4G cleavage, we have demonstrated that eIF4G-independent synthesis represents primarily *de novo* initiation as well. Insofar as cleavage of eIF4G completely disrupts cap-dependent translation initiation, *Xenopus* oocytes have the capacity to initiate translation on their resident mRNAs in a primarily cap-independent manner. Our findings are consistent with earlier observations that general protein synthesis in stage VI oocytes is resistant to inhibition by even millimolar concentrations of cap analogs (61). However, our data do not suggest a particular mechanism for the cap-independent initiation of endogenous oocyte mRNAs; one cannot assume that they are internally initiated in the fashion of IRES-containing RNAs (4).

We suggest that eIF4 factors preferentially stimulate the synthesis of a small subset of proteins. These are likely to be encoded by mRNAs with a strong cap dependence (62). For example, rapamycin inhibits insulin-stimulated phosphorylation of both PHAS-I and eIF4E in 32D cells containing the insulin receptor and IRS-1, reducing both eIF4E availability and affinity for mRNA, and this results in inhibition of insulin-stimulated Myc synthesis (63). The stimulation of general protein synthesis, however, is inhibited only 10% and actin synthesis, not at all. Overexpression of eIF4E, on the other hand, preferentially enhances the translation of mRNAs encoding growth- and cell cycle-related proteins such as Myc, ornithine decarboxylase, ornithine aminotransferase, bFGF, cyclin D1 and vascular proliferation factor (64–67). The idea that a small subset of mRNAs, which are specifically involved in cell proliferation, is more dependent on the cap recognition and unwinding machinery may explain the effects of eIF4E overexpression on growth phenotype. These cells undergo oncogenic tranformation and display rapid proliferation, both of which are reversible upon expression of antisense eIF4E RNA (68). Therefore, modulating the activity of eIF4 factors may regulate the translation of growth-related mRNAs without greatly altering the general protein synthetic activity of a cell. The 2A-induced cleavage of eIF4G causes similar inhibition of eIF4 factor activity which is functionally equivalent to eIF4E inhibition or depletion.

Our results suggest that mRNAs encoding 'housekeeping' proteins in meiotically arrested oocytes may be less cap dependent than those encoding growth-related proteins and that cap-dependent initiation is limited to favor the expression of housekeeping mRNAs. Stage VI oocytes remain arrested in the meiotic G_2 phase of the cell cycle for up to 3 months in the ovary without dividing, differentiating or proliferating (14). As a consequence of progesterone stimulation, the oocyte may selectively enhance cap-dependent initiation by increasing the activity or availability of the eIF4 factors. Increased phosphorylation and complex formation of eIF4 factors is observed upon hormone-induced meiotic maturation of *Xenopus* oocytes (69). Maturation also promotes the dissociation of FRGY2 from previously masked mRNAs (17) and the oocyte experiences a 2- to 3-fold stimulation in protein synthesis which involves the recruitment of maternal mRNAs, including those encoding c-Mos, cyclins A and B, and Cdk2, which are essential for cell cycle progression (24,70). Coincident with the recruitment of these mRNAs, they undergo cytoplasmic 3′ poly(A) elongation, which is also required for their mobilization into polyribosomes. Translation of an mRNA in *Xenopus* oocytes is enhanced by a poly(A) tail and there is evidence that the extended poly(A) tail facilitates reinitiation events and affects initiation events at the mRNA cap (71,72). The prediction which follows from these studies is that maternal mRNAs recruited during meiotic maturation in response to polyadenylation will be strongly cap dependent for that recruitment.

It is tempting to suggest that stage VI oocytes naturally utilize cap-independent translation for most of their mRNAs, but we cannot discount the possibilities that: (i) cap-independent synthesis is specifically induced by the cleavage of eIF4G, or (ii) another protein stably bound to endogenous mRNAs functionally replaces eIF4G. Possibility (i) is a quite attractive hypothesis, as it may ascribe a function to the cleavage products of eIF4G which result from the action of the protease. cp_N accumulates only transiently and is rapidly further degraded by cellular proteases, perhaps because it contains all of the significant PEST signals of the eIF4G molecule. Since it also contains the eIF4E binding site, the function served by eIF4E in 48S complex formation disappears with the degradation of cp_N. cp_C, on the other hand, is relatively stable in the oocyte after intact eIF4G has disappeared. cp_C contains the binding sites for eIF4A and eIF3 (and hence the 40S subunit), and it seems reasonable to suggest that such a complex might be competent to initiate translation on an mRNA in a cap-independent manner. Indeed, cp_C-containing ribosomes were recently shown to support cap-independent initiation in reticulocyte lysate (73). We observe that the synthesis of a few proteins (e.g. actin) from endogenous mRNA may even be enhanced by eIF4G cleavage. It has been suggested that highly efficient mRNAs like actin become less dependent on cap-mediated mechanisms once they are assembled into active polyribosomes (74). The enhanced synthesis of actin appears to correlate with stable accumulation of cp_C, consistent with the notion that stimulation of the cap-independent pathway is advantageous for actin mRNA.

As a system to assay the effects of eIF4G cleavage on cap-dependent and cap-independent initiation *in vivo*, *Xenopus* oocytes offer advantages over mammalian cells either infected with picornavirus or expressing protease 2A. The injection of recombinant protease 2A does not elicit the deleterious side-effects on transcription, eIF2 activity and cellular physiology which are observed during picornaviral infection of cells, allowing simpler interpretation of experiments. The warfare waged by both virus and host involving interferon response, dsRNA response, membrane disintegration, additional viral

proteases (e.g. 3C), RNA virus replication, IRES-driven translation, etc., does not complicate studies in oocytes. Because they are arrested in the cell cycle and transcriptionally silent, secondary effects on gene expression and cell proliferation can also be avoided. In such a system, where isolated effects on translation may be assayed *in vivo*, we find that eIF4G cleavage causes only minor inhibition of total protein synthesis, but severely inhibits cap-dependent initiation.

ACKNOWLEDGEMENTS

We thank Dr Tim Skern for the expression plasmid to produce recombinant CV2A and Dr Barry Lamphear and Ai-Li Cai for purified protease. Dr Raul Mendez, Dr Barry Lamphear, Weiniu Gan, Ming-Xing Gao, Vaishali Kereketta and Boyd Butler improved the manuscript with their comments and Dr Wolfgang Sommergruber provided helpful discussion. This study was supported by grant no. 3078 from the Council for Tobacco Research-USA, Inc. and grant no. GM20818 from the National Institute of General Medical Sciences.

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