Translocation of Globin Fusion Proteins across the Endoplasmic Reticulum Membrane in Xenopus laevis Oocytes

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Abstract. We have studied the translocation of a normally cytoplasmic protein domain across the membrane of the endoplasmic reticulum in cell-free systems and in Xenopus laevis oocytes. Coding regions for the normally cytoplasmic protein globin were engineered in frame either 3' or 5' to the coding regions for the signal sequence of either Escherichia coli b-lactamase or bovine preprolactin, respectively, in SP6 expression plasmids. RNA transcribed from these plasmids was microinjected into oocytes as well as translated in cell-free systems. We demonstrate that both in vivo and in vitro, a previously amino-terminal signal sequence can direct translocation of domains engineered to either side. Moreover, the domain preceding the signal sequence can be as large as that which follows it. While, in general, cell-free systems were found to faithfully reflect translocation events in vivo, our results suggest that a mechanism for clearance of signal peptides after cleavage is present in intact cells that is not reconstituted in cell-free systems.

Materials and Methods

Materials

Rabbit anti-human globin antisera was obtained from Cappel Laboratories,
Cochraneville, PA. Rabbit anti-ovine prolactin serum was from United StatesBiochemical Corp., Cleveland, OH. All restriction endonucleases, SP6
polymerase, T4 DNA ligase, and Klenow fragment of Escherichia
coli, and DNA polymerase I was from Boehringer Mannheim Diagnostics
Inc., Houston, TX, or from New England Biolabs, Beverly, MA. RNase
inhibitor was from Promega Biotec, Madison, WI. Trypsin, Trasylol, and
Proteinase K were from Boehringer Mannheim Diagnostics, Inc., Piscata-
way, NJ. Protein A-Sepharose was from Pharmacia Fine Chemicals, Piscata-
way, NJ. Xenopus laevis frogs were obtained from Nasco, Fort Atkinson,
WI. [35S]methionine was from New England Nuclear, Boston, MA; and
hydroxyurea was from Calbiochem-Behring Corp., Torrance, CA.

Constructions
All constructions were engineered into the pSP64 vector which was ob-
tained from Promega Biotec. A chimeric a-globin cDNA cloned into the
Pat site of pBRS22 was kindly provided by S. Liebhaber, University of
Pennsylvania School of Medicine, Philadelphia, PA. This plasmid, pMC18,
was cut with XmnI in the presence of ethidium bromide, followed by timed
digestion with Bal31, recut with Ncol, treated with Klenow fragment and
T4 DNA ligase. From the resulting transformants a perfect fusion of the lac-
tamase signal sequence with the initial methionine of globin was recovered
and subcloned into pSP64 as a Hind III-Pst I fragment. This plasmid was
 termed pSP125E. PSPGIE was derived from pSP125E by deletion of the sig-
nal sequence (open bar) and a portion of 5' untranslated region as a result
of cleavage with Bgl II, Nco I, treatment with Klenow fragment, and T4
DNA ligase. Details for the pSPGPII construction can be found in reference
14. PSPG2P was derived from pSPGPI by subcloning a BssH II-BstE II
fragment containing two globin coding regions into the BssH II-BstE II sites
of pSPGPI.

In Vitro Synthesis of Capped Transcripts
Cesium-purified plasmids were linearized at sites in the 3' untranslated
region, extracted in phenol/chloroform, ethanol precipitated, and dissolved in
water. Transcription was carried out in 10-ml volumes containing 2.5 µg lin-
erized DNA, 20 U SP6 polymerase, 0.25 mg/ml calf liver tRNA, 0.5 mM
ATP, 0.5 mM CTP, 0.5 mM UTP, 0.1 mM GTP, 0.5 mM GpppG caps),
10 mM dithiothreitol, 2 mM spermidine, 6 mM MgCl2, 40 mM Tris-Cl,
ph 7.5, and 0.9 U/ml human placental RNase inhibitor. Reactions were car-
ried out at 40°C for 1 h and aliquots of total reaction mix were used directly
for injection or in vitro translation.

Cell-free Translation
4-µl aliquots of total transcription products were added to translation reac-
tions of 20-µl vol containing 8.6 µl of rabbit reticulocyte lysate prepared as
described (II), 1 nCi/ml [35S]methionine, 0.2 mM each of the other 19
amino acids, methionine, 16 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM
MgCl2, 0.44 mM spermidine, 2 mM dithiothreitol, 0.9 mM GTP, 1 mM
ATP, 10 mM creatine phosphate, 0.4 mg/ml creatine phosphokinase, 0.1
mg/ml calf liver tRNA. Reaction mixtures were incubated at 24°C for 60
min in the absence or presence of 2 U/ml dog pancreas rough microsomes
(prepared as described, reference 24).

Posttranslational Protease Protection
After a 1-h incubation at 24°C in the presence of membranes, translation
reaction mixtures were chilled on ice, adjusted to 10 mM CaCl2, and
divided into equal aliquots of 5 or 10 µl. Some were treated with proteinase
K (dissolved in 10 mM CaCl2, 50 mM Tris (pH 7.5), and preincubated at
37°C for 15 min) at a final concentration of 0.3 mg/ml either in the presence
or absence of 1% Nickol (a nonionic detergent used to disrupt the lipid
bilayer). Proteinase digestion was stopped by the addition of 2 mM
phenylmethylsulfonyl fluoride (PMSF) and samples were immediately
transferred to 4-5x vol 1% SDS in 0.1 M Tris-HCl (pH 8.9) preheated to
100°C, then incubated at 100°C for 10-15 min. Samples were diluted 20-
fold in a solution of 1% Triton, 0.1 M Tris-HCl (pH 8.0), 10 mM EDTA,
100 mM NaCl, subjected to immunoprecipitation with either 0.5 µl
anti-prolactin antiserum or 4 µl anti-globin antiserum and protein A-Sepha-
rose CL4B, followed by SDS PAGE.

Microinjection of Xenopus Oocytes
Xenopus oocytes were manually dissected and were subsequently injected
and labeled in modified Barth's saline solution (MBSH) containing
[(35S]methionine as described (6, 20). MBSH contains 88 mM NaCl,
1 mM KCl, 2.4 mM NaHCO3, 0.82 mM MgSO4, 0.33 mM Ca (NO3)2,
0.41 mM CaCl2, 10 mM Hepes (pH 7.6).

Immunoprecipitation of Oocyte Translation Products
Medium and oocytes were separated, and the medium was then diluted in
4x vol Buffer A (1% Triton X-100, 0.1 M Tris-HCl (pH 8.0), 0.1 M NaCl,
0.01 M EDTA, 1 mM PMSF); oocytes were homogenized in 20 µloocyte
of Buffer A. 2-10 µl antiserum was added to each sample, and samples were
incubated overnight at 4°C. After incubation, samples were centrifuged in
an Eppendorf centrifuge at 10,000 g for 1 min to remove aggregates, and
the supernatants were then transferred to a fresh tube containing 15 µl of
a 50% slurry of protein A-Sepharose in Buffer A. Samples were incubated
for 1 h at 4°C, washed three times with Buffer A, and twice subsequently
with buffer containing 0.1 M Tris-HCl (pH 7.5) and 0.1 M NaCl to remove
residual Triton.

Figure 1. Line diagrams of relevant regions of SP6 expression plas-
mids. Restriction maps of plasmids are indicated above the lines and
defined coding regions by the bars below. The SP6 promoter is indicated
d by the small solid black box, denoted SP6p. pSPGIE encodes full-length chimpanzee a-globin (143 amino acids; open
bar). PSPBP3 encodes full-length bovine preprolactin; the signal
sequence is represented by the solid black bar and mature prolactin
joined to the initial methionine of full-length chimpanzee a-globin (open bar). PSPGPI encodes a fusion protein of the first 109 amino acids of chimpanzee
a-globin (open bar) flanked on the amino terminus by globin and on the carboxy termi-
nus by mature prolactin (cross-hatched bar). pSP125E encodes a perfect fusion of the
3-lactamase signal sequence (stippled bar) joined to the initial
methionine of full-length chimpanzee a-globin (open bar). PSPGP1 encodes a fusion protein of the first 109 amino acids of chimpanzee
a-globin (open bar) followed by full-length bovine preprolactin such that the signal sequence of preprolactin (solid black box) is
flanked on the amino terminus by globin and on the carboxy termi-
nus by mature prolactin (cross-hatched bar). pSPG2P is identical to
pSPGPI except that it encodes the first 109 amino acids of globin fused to amino acids 21-109 of globin (open bar) followed by full-
length preprolactin. In subsequent figures, the full-length encoded
products are described with the prefix "pre" (e.g., pre-GSP and pre-
G2SP), and the products of signal cleavage are described according to
their corresponding coding regions (e.g., P1, GSI, and G2SI).
Cell-free translation of pSPI25E. Transcription-linked wheat germ translations programmed with pSPI25E were carried out as described in Materials and Methods. Reactions were carried out either in the absence (lanes A and B), or presence (lanes C–E) of dog pancreas rough microsomes. Some samples were subsequently treated with trypsin in the absence (lane D) or presence (lane E) of 1% Triton. Samples were immunoprecipitated with anti-globin antiserum (lanes B–E), or nonimmune serum (lane A), subjected to SDS PAGE, and viewed by autoradiography. Downward-pointing arrowheads indicate the signal sequence–containing precursor, pre125E; upward-pointing arrowheads indicate the processed molecule, 125E, from which the signal sequence has been cleaved.

Pulse-Chase

Oocytes were injected with in vitro-synthesized transcripts, preincubated for 5 h, and pulse-labeled for 1 h in MBSH containing 5 mCi/ml 3SS-met. Labeled medium was then removed, oocytes were briefly washed in cold MBSH, and then incubated for varying lengths of time in MBSH containing 10% fetal calf serum and 20 mM unlabeled methionine.

Subcellular Fractionation of Injected Oocytes

Freshly labeled oocytes were homogenized with 40 μl/oocyte at 4°C in an isoosmotic buffer containing 10% (wt/vol) sucrose, 150 mM NaCl, 10 mM MgAc, 20 mM Tris–HCl (pH 7.6) (Buffer B). 500 μl of total homogenate was layered over a 1-ml cushion of 20% sucrose (wt/vol), 50 mM NaCl, 10 mM MgAc, 20 mM Tris–Cl (pH 7.5) (Buffer C), and spun at 15,000 g for 30 min in the ultracentrifuge. The top 500 μl (cytosolic components) was removed, the rest of the supernatant aspirated, and the pellet (vesicles) resuspended in Buffer A.

Proteolysis of Oocyte Vesicles

Freshly labeled oocytes were homogenized with a ground glass homogenizer in 40 μl/oocyte of Buffer B. Each sample was divided into three aliquots: the first aliquot was a control with no added protease, and the other two aliquots were treated with 0.3 mg/ml Proteinase K in the presence or absence of 1% Triton X-100. All samples were incubated for 3 h at 4°C. Protease digestion was stopped by the addition of 2 mM PMSF followed by boiling in 2× vol 2% SDS in 0.1 M Tris-Cl (pH 8.9) for 15 min. Samples were then diluted 20-fold in a solution of 1% Triton, 0.02 M Tris (pH 8.0), 2 mM EDTA, 20 mM NaCl, and subjected to immunoprecipitation followed by gel electrophoresis.

Results

We have previously shown that the lactamase signal sequence together with the first five amino acids of authentic lactamase is sufficient to direct globin to the lumen of microsomal vesicles in cell-free systems (9). We initiated the present study by extending these initial observations using a construct encoding a perfect fusion between the signal and globin coding regions placed behind the SP6 promoter (pSPI25E).

Fig. 1 displays line diagrams of the relevant coding regions for plasmid pSPI25E and other plasmids studied here. RNA was transcribed from this plasmid using SP6 polymerase, and was translated in a wheat germ cell-free system in the...
absence and presence of microsomal membranes prepared from dog pancreas. In the absence of membranes, a single globin-immunoreactive product (termed pre125E), which was of higher molecular weight than authentic globin, was observed (Fig. 2, lane B). In the presence of membranes, an additional globin-immunoreactive band (termed 125E), which co-migrated with authentic globin, was observed (Fig. 2, lane C). To determine the localization of these two protein species, proteases were added after completion of translation. Pre125E was localized outside of the microsomal vesicle (i.e., in the topologic equivalent of the cytosol), since it was degraded by added proteases. However, 125E was quantitatively protected from protease (Fig. 2, lane D) unless nonionic detergent was added to solubilize the protecting lipid bilayer (Fig. 2, lane E), thus indicating the microsomal vesicle lumen as its location.

To determine the subcellular localization of 125E in vivo, transcripts were microinjected into Xenopus oocytes. Control oocytes were co-injected with transcripts encoding a cytosolic protein (GIE, authentic full length globin without a signal sequence) and a normally secreted protein (BP3, full-length preprolactin). Fig. 3a shows that after subcellular fractionation, GIE was localized to the cytosol (lanes A and B), whereas BP3 was in the vesicle fraction (lanes C and D). When injected oocytes were homogenized in an isosmotic buffer and subsequently incubated with proteinase K, GIE was completely degraded (Fig. 3a, lanes E-G), whereas BP3 was completely protected from digestion (Fig. 3a, lanes H-J). When pSP125E transcripts were injected into Xenopus oocytes, a single globin-immunoreactive product was observed after a 1-h pulse with [35S]methionine (Fig. 3b, lane A). Neither nonimmune control precipitations (Fig. 3b, lanes B and D), nor anti-globin immunoprecipitation of mock-injected oocytes (Fig. 3b, lane D) demonstrated globin-immunoreactive products under these conditions. This product, which co-migrated with 125E, was demonstrated by cell fractionation to be located quantitatively in the vesicle fraction of the oocyte (Fig. 3b, lanes E and F). Moreover, proteolysis demonstrated that, like its cell-free counterpart (see Fig. 2), the mature 125E product was localized to the vesicle lumen (Fig. 3b, lanes G-J). We conclude that both in intact cells and in cell-free systems, an amino-terminal signal sequence is sufficient to direct a normally cytoplasmic protein across the endoplasmic reticulum membrane. Elsewhere we will demonstrate that this mature globin localized to the ER lumen can be transported through the secretory pathway and secreted into the medium (Simon, K., and V. R. Lingappa, manuscript in preparation).

Having established that a simple substrate displays similar translocation behavior in vitro and in vivo, we studied more complex constructions. Recently, we engineered a globin coding region 5′ to the normally amino-terminal signal sequence of bovine prolactin. Plasmid pSPGPl encodes a fusion protein comprised of the initial 109 amino acids of globin followed in frame by the signal sequence (30 amino acids) and the entire coding region of bovine prolactin (199 amino acids). When pSPGPl was expressed in a cell-free transcription–translation system, the former amino-terminal prolactin signal sequence still directed translocation of the prolactin domain with concomitant cleavage of the signal sequence (15). Moreover, the globin domain flanking the signal on the amino terminus was also translocated, albeit with apparent reduced efficiency.

To extend these studies, we constructed a similar chimeric protein in which part of a second globin-coding region was engineered into that of pSPGPl. This new plasmid, pSPG2P, encoded 250 amino acids of globin (parts of two globin-coding regions arranged in tandem) followed by the prolactin signal sequence (30 amino acids) and mature prolactin (199 amino acids) (see Fig. 1). When expressed in the transcription-linked translation system from reticulocyte lysate, a single polypeptide of 45,000 D, termed preG2SP, was observed (Fig. 4, lane A); this protein was both globin and prolactin immunoreactive (Fig. 4, lanes A, C, and F). When membranes were present during translation, two new products were observed. One of these membrane-dependent products, termed G2S1, was globin but not prolactin immunoreactive, and this peptide was the predicted size for the globin domains joined to the signal sequence (Fig. 4, lane C). The other product, termed PI, was prolactin but not globin immunoreactive, and it co-migrated with mature bovine prolactin (Fig. 4, lane F). Digestion with protease revealed that, as observed previously with GS1 (15), some, but not all, G2S1 chains were protected from protease in the absence of detergent (Fig. 4, lane D). In the same aliquot, essentially all PI chains were protected from protease in the

Figure 4. Cell-free translation of pSPG2P. pSPG2P was used as a template for SP6 polymerase and the resulting RNA was used to program cell-free translation in rabbit reticulocyte lysate. Translation reactions were carried out in the absence (lanes A and B) or presence (lanes C–H) of dog pancreas microsomes. Aliquots of translation product were incubated with proteinase K (lanes B, D, E, G, and H), some with Triton added (lanes E and H); or with no additions (lanes A, C, and F) as described in Materials and Methods. Samples were immunoprecipitated with anti-prolactin (lanes A, B, F-H) or anti-globin (lanes C-E) serum and analyzed by SDS-PAGE followed by autoradiography. Downward-pointing arrowheads indicate the precursor, preG2SP. Small upward-pointing arrowheads in lanes C and D indicate G2S1 and in lanes F and G indicate PI. The dot alongside lane D denotes minor contaminants of PI in samples immunoprecipitated with anti-globin serum.
A and C) or anti-globin (Fig. 5, lanes B and D) serum and pSPG1 and pSPG2 protein included full-length precursor (pre-
tin, see Fig. 3), the products expressed in oocytes from both
globin fragments generated from cleavage of the signal sequence in
lanes A and B.

absence of detergent (Fig. 4, lane G). Thus, enlarging the
domain preceding the signal sequence appeared to have little
effect on either promoting or preventing translocation of that
domain. These results have been reconfirmed by using glycosylated domains (15).

These results suggest that translocation of a single globin
domain engineered 5' to a signal sequence was not a fortui-
tous result limited to domains of a narrow range of sizes. To
determine whether the unusual translocation events which
were consistently observed in cell-free systems also occurred
in intact cells, pSPG1 and pSPG2 protein transcripts were ex-
pressed in oocytes (data not shown). Thus, when a previ-
ous location, both flanking domains can be translocated
across the endoplasmic reticulum membrane in cell-free sys-
tems and in vivo.

In contrast to our results in cell-free systems, where the
G1 (15) and G2S1 chains (Fig. 4) were generated in a 1:1
to PI, much less GS1 and G2S1 than PI was detected
protected. Similar cell fractionation and proteolysis
results were obtained when transcripts of pSPG1 were ex-
pressed in oocytes (data not shown). Thus, when a pre-
viously amino-terminal signal sequence is engineered to an
ternal rather than the initial AUG. To test this possibility, in-
jected oocytes were incubated in the presence of hydroxyleu-
Figure 7. Proteolysis of G2P in Xenopus oocytes. Groups of 15 oocytes were injected with pSPG2P transcripts and then labeled for 6 h with [35S]methionine as described. Labeled oocytes were homogenized in isosmotic buffer and digested with proteinase K in the absence (lanes B and E) or presence (lanes C and F) of 1% Triton; lanes A and D show total products in the absence of proteolysis. Each sample was then divided into two aliquots, and one set was immunoprecipitated with anti-prolactin (lanes A-C), and the other with anti-globin (lanes D-F) serum. Samples were analyzed by SDS PAGE and viewed by autoradiography. Downward-pointing arrowheads refer to G2SP; upward-pointing arrowheads refer to chains migrating to the position of authentic prolactin (lanes A-C) or to immunoreactive globin fragments (lanes D-F). Equivalent amounts of oocyte products were used for lanes A-C and D-F; however, the former lanes were from a shorter exposure, to avoid overexposure of the prolactin-reactive bands.

Results of a pulse-chase experiment in which all products generated from both fusion proteins were followed through time. Whereas the amounts of precursor and mature prolactin (localized to the cytosol and secretory pathway, respectively) remained relatively constant, both the GS1 and G2S1 chains were rapidly degraded with a half-time of ≈30 min. Consistent with these observations, P1 (but not GS1 or G2S1 fragments) can be detected in the incubation medium after a long chase (data not shown). At least one event (rapid degradation), apparently specific for cleaved signal sequences and their associated domains is not reconstituted in our cell-free systems. We suggest that this process, detected by the use of engineered probes with lengthened signal-associated domains, may reflect the normal metabolism of signal sequences after cleavage.

Discussion

We initiated these experiments for several reasons. First, we wished to extend our earlier results which suggested that, when placed in an internal location, a previously amino-terminal signal sequence could direct the translocation of both amino- and carboxy-terminal domains. In the present

cine, a leucine analogue which is incorporated into nascent chains; the presence of hydroxyleucine prevents translocation and signal cleavage by inhibiting the interaction of the signal sequence with SRP (8, 25). If signal cleavage of GP1 and G2P were abolished, this should result in the disappearance of P1 and the appearance of the true precursor from which the P1 chains were generated. Fig. 8 shows the results of this experiment. Injection of hydroxyleucine into oocytes that had been preincjected with pSPGP1 or pSPG2P transcripts abolished the P1 product and resulted in accumulation of larger amounts of the precursors, preGSP and preG2P; no additional products were observed (Fig. 8, lanes C-F). Similar treatment of oocytes expressing bovine preprolactin transcripts resulted in disappearance of mature prolactin and an accumulation of preprolactin (Fig. 8, lanes A and B). Therefore, the nonstoichiometry of G2S1 or GS1 and P1 observed in oocytes was not a result of initiation at an internal site.

An alternative explanation for the disproportion between P1 and GS1 or G2S1 chains detected is that an equivalent number of prolactin and globin-containing domains were initially generated, and that the GS1 and G2S1 chains were selectively recognized due to the presence of the cleaved signal and were subsequently degraded. Fig. 9 shows the
study, we show that this phenomenon occurs even when the domain which precedes the signal is the same size as that which follows it. Second, we wished to corroborate these surprising findings in vivo in order to rule out the possibility that the observed translocation reflected a permissiveness unique to cell-free systems. Results presented here demonstrate that such internalized signal sequences can function to translocate both flanking domains in intact cells. It should be noted that the experiments presented here show yields and not rates of assembly, since translocation into the endoplasmic reticulum can occur posttranslationally (16, 17, 26).

In addition to addressing these questions, this study yielded some unexpected findings. Cell-free systems typically yield incomplete conversion of nascent precursors to processed authentic chains. This is in contrast to the lack of observed precursor in living cells and tissues and has been presumed to reflect inefficiency of signal–receptor recognition in a fractionated, heterologous system. Consistent with this interpretation, increase in the concentration of available receptors (by adding increasing membrane concentration) results in increased efficiency of translocation in vitro (10). Investigation of these phenomena in *Xenopus* oocytes has been revealing. We have detected precursors in oocytes, and this observation establishes the *Xenopus* oocyte as an in vivo system in which probes of translocation might be studied with greater sensitivity by measuring changes in the relative amounts of precursor and products which accumulate. Thus, it might be possible to approach directly such previously inaccessible problems as the role of SRP-mediated elongation arrest in vivo by microinjection of SRP.

The finding that signal-bearing cleavage fragments were grossly under-represented in oocytes was at variance with the results from cell-free systems. Since full-length precursor and full-length globin appeared reasonably stable in the cytosol, and processed I25E (globin), as well as mature prolactin, appear stable in the endoplasmic reticulum lumen and secretory pathway, we suspect that the extremely rapid degradation of the processed signal-containing globin domains reflects a fate peculiar to signal-containing cleavage fragments. However, we cannot exclude the possibility that the instability of GS1 and G2SI may be caused by their inability to be completely translocated across the endoplasmic reticulum membrane.

Signal sequences are generated stoichiometrically with secretory and membrane proteins, and in some tissues specialized for secretion (pituitary or pancreas, for example) the quantities of stored secretory product constitutes a large percentage of total cell protein, thus it seems likely that an efficient mechanism for clearance of cleaved signal sequences might exist. Attempts to study the fate of cleaved signal peptides have been hampered by the difficulty in detecting the small, hydrophobic, cleaved peptide. For this reason, the true fate of cleaved signal sequences has remained largely an unsolved problem. By joining a passenger domain to the amino terminus of a signal sequence, the size of the resultant cleaved, signal-bearing domain has been greatly increased. Moreover, the globin domain now serves to identify the fragment such that it can be immunoprecipitated and easily detected by standard SDS PAGE. While we cannot rule out the possibility that such engineering has altered the fate of the cleaved signal itself, the distinctive kinetics of degradation of the two different signal-bearing cleavage fragments compared to even the same domains when part of a full-length precursor suggests that the rapid degradation of signal-bearing globin fragments occurred by a mechanism normally used for signal degradation. Our findings provide the basis for an assay in which we can attempt to localize the signal clearance machinery by reconstituting these events in vitro using oocyte cell fractions.

To date, most progress in defining the mechanisms of targeting to and translocation across the endoplasmic reticulum membrane derives from studies in cell-free systems. While the results of this study reconfirm that the translocation behavior of altered substrates in cell-free systems is largely faithful to the corresponding events in vivo, our results identify at least one translocation event which cannot be studied in currently available cell-free systems; namely, the fate of cleaved signal sequences. *Xenopus* oocytes programmed with the engineered substrates described here and in conjunction with appropriate antibodies should permit elucidation of this issue. Studies are in progress to exploit these and other implications of engineered substrate function in *Xenopus* oocytes.

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Simon et al. *Translocation of Proteins in X. laevis oocytes*
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