Misfolding and Aggregation of Newly Synthesized Proteins in the Endoplasmic Reticulum

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Abstract. As a part of our studies on the folding of glycoproteins in the ER, we analyzed the fate of viral glycoproteins that have misfolded either spontaneously or through inhibition of N-linked glycosylation. Newly synthesized Semliki Forest virus spike glycoproteins E1 and p62 and influenza hemagglutinin were studied in infected and transfected tissue culture cells. Misfolded proteins aggregated in < 1 min after release from polysomes and aberrant interchain disulfide bonds were formed immediately. When more than one protein was misfolded, mixed aggregates were generated. This indicated that the formation of complexes was nonspecific, random, and not restricted to products from single polysomes. The size of the aggregates varied from small oligomers to complexes of several million daltons. BiP was associated noncovalently with the aggregates and with some of the nonaggregated products. We conclude that aggregation reflects the poor solubility of incompletely folded polypeptide chains.

THE most common reason for the failure of denatured proteins to fold correctly during in vitro refolding experiments is irreversible aggregation due to the inherent insolubility of partially and incorrectly folded polypeptide chains (see Jaenicke, 1987). Apparently, such aggregation is not restricted to in vitro conditions. It is also a problem during protein folding in living cells. Aggregation of newly synthesized proteins occurs in the cytoplasm of bacteria which accumulate misfolded and incompletely folded proteins in so-called inclusion bodies (Shively, 1974; Williams et al., 1982; Marston, 1986; Haase-Pettingell and King, 1988). It occurs in the ER of cells synthesizing misfolded proteins (Gibson et al., 1979; Hurtley et al., 1989) and may be the underlying defect in several diseases where proteins accumulate in this organelle (Carlson et al., 1989; Cheng et al., 1990).

In the ER, aggregates can form both between soluble proteins as well as between membrane-bound proteins (Gibson et al., 1979; Kornfeld and Kornfeld, 1985; Machamer and Rose, 1988b; Hurtley et al., 1989; Tooze et al., 1989; Singh et al., 1990; Machamer et al., 1990). Aggregate size ranges from large inclusion bodies to small oligomers. Aggregates often contain aberrant disulfide crosslinks and BiP/GRP78, an abundant ER chaperon, is generally a noncovalently associated component. The aggregated proteins are retained in the ER and often slowly degraded over several hours. When large amounts of misfolded proteins accumulate, the ER compartment dramatically increases in size to accommodate the increased load of transport-incompetent protein (Pacifici and Iozzo, 1988; Tooze et al., 1989; Carlson et al., 1989).

To understand the mechanisms of protein folding and quality control in the ER, we analyzed the formation of aggregates between viral spike glycoproteins. In previous studies, we showed that nonglycosylated and misfolded influenza hemagglutinin (HA)[1] covalently aggregated rapidly after completion of synthesis (Hurtley et al., 1989). The aggregation is much faster than the normal homotrimerization of HA which occurs with a $t_{1/2}$ of ∼10 min (Copeland et al., 1986; Gething et al., 1986).

The difference in rate between aggregation and normal trimerization, which happens randomly from a mixed pool of monomers (Boulay et al., 1988), raised the possibility that aggregation might occur at the level of individual polysomes. To test this, we analyzed the aggregation products in cells where two or more viral proteins were synthesized simultaneously. We found that these proteins formed mixed aggregates whether synthesized from the same polysome or from different polysomes. Our results indicate that aggregation constitutes a general, nonspecific precipitation reaction which is not limited to products from a single polysome.

Materials and Methods

Cells

HeLa cells were grown in MEM with 10% FCS. COS cells were grown in DME with 10% FCS and 10 mM Hepes, pH 7.3.

Cloning of SFV- and Influenza cDNAs

The cDNAs for E1 and p62 were taken from plasmids obtained from H. Garoff (Department of Molecular Biology, Karolinska Institute, Huddinge, Sweden) (Melancon and Garoff, 1986; Cutler and Garoff, 1986). For expression in HeLa cells, both cDNAs were recloned into pGEM 3 (Promega, Madison, WI) and expressed from the T7 promoter. The E1 construct en-

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1. *Abbreviations used in this paper:* HA, hemagglutinin; PCR, polymerase chain reaction; SFV, Semliki Forest virus.

codes the full-length E1 protein and contains a 400-bp sequence at the 5' end representing the cDNAs for the full 6-kD protein and minor parts of the capsid and E2 sequences. The cDNA of the p62 construct was modified by polymerase chain reaction (PCR)[1] to eliminate the rest of the 6-kD sequence and codes for the full-length capsid and p62 proteins. PCR was also used to introduce a different 5' modification of the same cDNA to allow cloning into a different expression vector (pCite; Novagen, Madison, WI). This vector uses the untranslated region of encephalomyocarditis virus to enhance translation efficiency (Elroy-Stein et al., 1989).

The cDNA for the X31 influenza hemagglutinin was obtained from a recombinant virus described earlier (Doms et al., 1986) and cloned into Bluescript KS+ (Stratagene, La Jolla, CA) under T7 promoter control. The entire cDNA was resequenced. The clone has a single point mutation compared to the published base sequence (Verhoeyen et al., 1980) which converts amino acid 132 in HA2 (Glu) into asparagine. This mutation occurs naturally in closely related strains of influenza A (Verhoeyen et al., 1980).

Transfection

COS cells (Gluzman, 1981) were transfected using the DEAE-dextran method (Sompayrac and Danna, 1981; Luthman and Magnusson, 1983) with plasmids containing the E1 or p62 cDNAs, respectively (Melancon and Garoff, 1986; Cutler and Garoff, 1986).

HeLa cells were transfected with the vector constructs described above using liposomes containing the cationic synthetic lipid dimethyldioctadecylammonium bromide (Felgner et al., 1987; Rose et al., 1991) and expressed using a recombinant vaccinia virus synthesizing T7 RNA polymerase (Fuerst et al., 1986). Cells were infected with the recombinant vaccinia virus in serum-free DME for 30 min (multiplicity of infection = 50). Each dish was then incubated with the plasmid-liposome mixture in DME for several hours. The liposomes were the generous gift of J. K. Rose (Department of Pathology, Yale University School of Medicine, New Haven, CT) (Rose et al., 1991) and were later obtained from a commercial source (TransfectAce; BRL, Gaithersburg, MD). The DNA-liposome ratio was optimized for maximal transfection efficiency. A 60-mm dish typically received 10 μg of each plasmid and 10 μl TransfectAce. For each experiment, double transfection efficiency was controlled by transfecting cells on coverslips with the same mixtures and detecting the expressed proteins by immunofluorescence with antibodies against E1 and p62.

As described earlier for a different cell system (Felgner et al., 1987), the transfection time was of great importance for the liposome-mediated transfection rate. The term transfection time will be used to refer to the time from the beginning of the incubation with the liposome-DNA mixture to the time point where the cells were lysed. After a transfection time of 6 h, >90% of the cells expressed both proteins. Decreasing this time period to 90 min decreased the transfection rate to 30-40% with 60-70% of these cells expressing both proteins.

Infection with SFV

Cells were infected with SFV (multiplicity of infection = 100) for 1.5 h in RPMI 1640 (Gibco Laboratories, Grand Island, NY), 0.2% BSA, 10 mM Hepes, pH 6.8, at 37°C, and then incubated for 5 h in DME with 10% FCS.

Inhibition of Glycosylation Using Tunicamycin

At the end of the infection or liposome incubation, cells were preincubated for 45 min in methionine-free medium containing 5 μg/ml tunicamycin (stock solution: 10 mg/ml tunicamycin in DMSO). The drug was also present during the pulse and chase periods. Control cells were treated with an equivalent concentration of DMSO.

Metabolic Labeling and Cell Lysis

Metabolic labeling was performed as described earlier (Braakman et al., 1991). To avoid artificial disulfide bond formation during cell lysis, cells were washed and lysed in the presence of 20 mM N-ethylmaleimide as previously described (Braakman et al., 1991).

Antibodies

The polyclonal anti-SFV antibody was prepared against spike complexes. It precipitates E1, E2, and p62 quantitatively and a small amount of capsid protein. Affinity-purified polyclonal antibodies against E1 and p62 were a gift of H. Garoff (Karolinska Institute) (Ziemiecki and Garoff, 1978) and

characterized earlier (Kielian and Helenius, 1985). The anti-p62 antibody precipitates p62 and its cleavage product E2 equally well. The polyclonal antibody against X31 influenza hemagglutinin was described before (Doms et al., 1985). *Staphylococcus aureus* complexes with the antibodies were washed twice in 0.05% Triton X-100, 0.1% SDS, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.6, at room temperature.

The monoclonal anti-BiP/GRP78 antibody was a gift of D. Bole (Howard Hughes Medical Institute, Ann Arbor, MI) (Bole et al., 1986). For immunoprecipitations with this antibody, cells were lysed in the presence of apyrase (30 U/ml), and the lysates were kept between 0 and 4°C. *S. aureus* complexes with the antibody were washed twice in 100 mM Tris-HCl, 0.5 M NaCl, pH 8.0, and once in 30 mM Tris-HCl, 20 mM MES, 100 mM NaCl, 0.5% Triton X-100, pH 7.5.

In Vitro Translation

The vector construct containing the E1 cDNA was transcribed in vitro with T7 RNA polymerase for 90 min at 37°C. The mRNA was precipitated and translated in a reticulocyte lysate system for 60 min at 30°C in the presence or absence of dog pancreas microsomes. Half of that mixture was then digested for 60 min on ice with 50 μg/ml proteinase K in the presence of 2 mM CaCl₂. After adding PMSF to a final concentration of 3 mM and incubating on ice for 5 min, the treated and untreated samples were reduced and analyzed by SDS-PAGE without prior immunoprecipitation.

Results

Mixed Aggregates in Semliki Forest Virus-infected Cells

Aggregate formation in the ER was initially studied in HeLa cells infected with Semliki Forest virus (SFV). The structural proteins of this Toga(alpha)virus are synthesized as a polyprotein which is cotranslationally cleaved into four polypeptides (see Garoff et al., 1982). Of these, the two glycoproteins p62 (62 kD) and E1 (50 kD) constitute type I membrane proteins with large lumenal domains. p62 has three N-linked sugars and 18 cysteines in the ectodomain, while E1 has one N-linked sugar and 16 cysteines. Soon after synthesis and translocation into the ER, p62 and E1 form heterodimers and move via the Golgi complex to the cell surface. In transit through the Golgi network or in the secretory vesicles, p62 is cleaved to E2 (49 kD) and E3 (10 kD).

When lysates made from [³⁵S]methionine-labeled, SFV-infected cells were immunoprecipitated with an antibody against the spike glycoproteins, the proteins observed in SDS-PAGE were p62, the mature E2, E1, a 97-kD protein representing untranslocated polyprotein (Garoff et al., 1978), and a small amount of capsid (C) protein (Fig. 1, lanes *1* and *2*). E1 and E2, which separate well on nonreduced gels, comigrate after reduction (lane 2). The various protein bands in Fig. 1 and subsequent figures have been marked by subscripts "ox" and "red" to indicate whether the proteins are reduced or not, and with superscript "ng" in case the proteins are nonglycosylated.

In the nonreduced samples, aggregates were seen at the interface between the stacking and separating gels and on top of the stacking gel (Fig. 1, lane *1*, agg.). They represent disulfide crosslinked spike glycoproteins which are dissociated in SDS after reduction (Ziemiecki et al., 1980). Their presence indicated that partial misfolding of p62 and E1 molecules occurred spontaneously in infected cells. The interchain disulfide bonds in these crosslinked aggregates, and others observed in subsequent experiments, were not formed after lysis by air oxidation, because our protocol included an alkylation step both before and during lysis of the cells. We have previously shown that alkylation of free sulf-
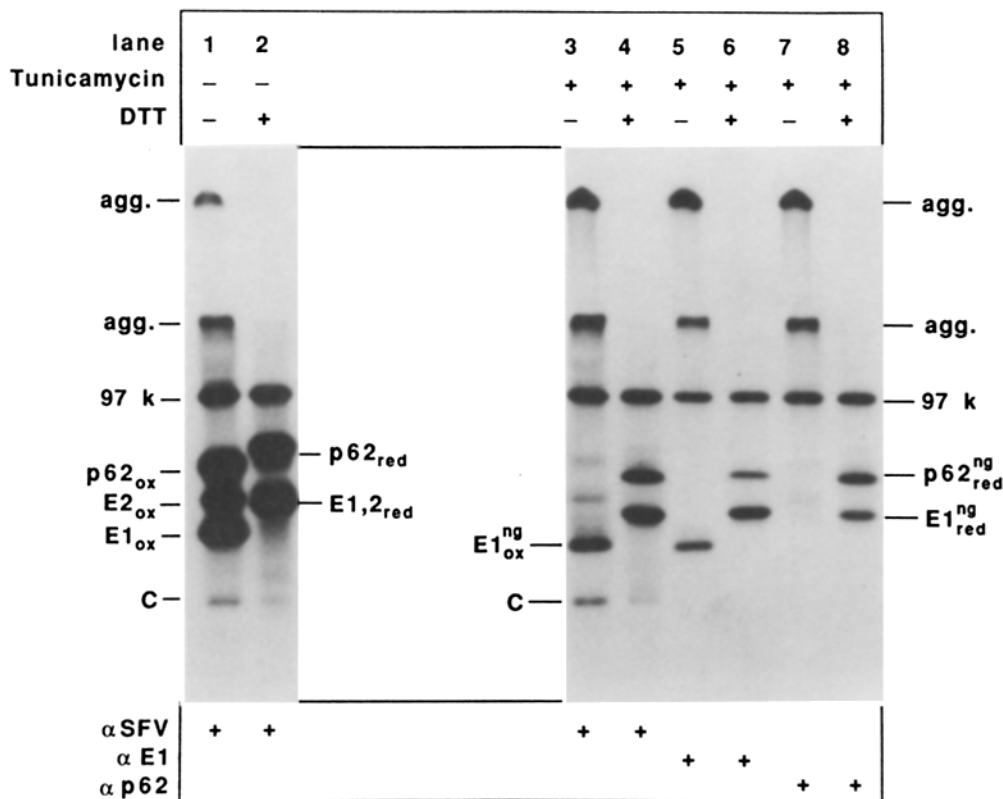
Figure 1. Tunicamycin-induced aggregate formation in SFV-infected cells. HeLa cells were infected with SFV as described in Materials and Methods. 6.5 h after the start of the infection cells were starved for methionine for 45 min in the presence or absence of 5 μg/ml tunicamycin. After a radioactive pulse for 30 min the cells were chased for 30 min and lysed as described. In the tunicamycin-treated cells the drug was present during the pulse and chase. Immunoprecipitations were done with the polyclonal antibody against SFV-spike glycoproteins (αSFV) or with antibodies specific for E1 ($\alpha E1$) or p62/E2 ($\alpha p62$), respectively. SDS-PAGE of nonreduced ($-DTT$) and reduced ($+DTT$) immunoprecipitates is shown. *agg*, aggregates; *C*, capsid; *ng*, nonglycosylated.

hydryl groups inhibits formation of artificial disulfides in cell lysates (de Silva et al., 1990).

When tunicamycin was added to inhibit N-linked glycosylation (Tkacz and Lampen, 1975), almost all of the labeled p62 and ∼50% of the E1 was recovered in aggregates (Fig. 1, lane *3*). The rest of the E1 migrated in the SDS gel as monomers ($E1_{ox}^{ng}$). The increased aggregation of p62 and E1 was consistent with the observation that most glycoproteins need their N-linked sugars to fold correctly (for review see Olden et al., 1982). Upon reduction, the aggregate bands were lost and the p62 and E1 bands were correspondingly stronger (lane *4*). Since the proteins lacked their N-linked sugars, they migrated faster than their glycosylated counterparts (cf. lane *2*).

Precipitation with antibodies which recognized either E1 or p62 alone demonstrated that the tunicamycin induced aggregates were mixed. Anti-E1 antibodies precipitated aggregates which contained both $E1_{red}^{ng}$ and $p62_{red}^{ng}$ (Fig. 1, lane *6*), and the same was seen for anti-p62 antibodies (lane *8*). Since the anti-E1 antibody also precipitated the nonaggregated form of E1 (lane *5*) relatively more E1 was precipitated than with anti-p62 (compare lanes *6* and *8*). We concluded that the nonglycosylated membrane polypeptides tended to form disulfide bonded aggregates and that these aggregates contained a mixture of p62 and E1.

Mixed Aggregates in Transfected Cells

Since E1 and p62 were derived from the same polysomes and therefore translocated in close proximity to each other, it was not surprising that they would end up in the same aggregates. What would happen if they were synthesized from different polysomes?

To test this, cDNAs of p62 and E1 were cloned into separate vector constructs as described in Materials and Methods. When expressed in vaccinia-infected HeLa cells, both proteins showed the expected molecular weights and the correct gel mobility shifts upon reduction (Fig. 2). In cotransfected cells most of the E1 and p62 dimerized and reached the *trans*-Golgi network as judged by the cleavage of p62 to E2 (not shown). No spontaneous disulfide-linked aggregates were observed if the radioactive pulse was given 2 h after the beginning of the transfection. Extensive spontaneous aggregation of all studied proteins (E1, p62, and HA) was, however, seen after longer transfection times. This aggregation was independent of the expression level of the proteins and obviously reflects major vaccinia induced cytopathic effects on the folding of glycoproteins in the ER. We observed for p62 that the spontaneous aggregation after 6 h of transfection was highly temperature dependent: no aggregates were found at 32°C, whereas up to 50% of the proteins misfolded and aggregated at 37°C when expressed at a similar level (not shown).

Immunoprecipitations from lysates of E1-transfected cells showed variable amounts of a band at 60 kD which retained its position upon reduction (Fig. 2, *p*). Proteinase digestion experiments after in vitro translation in the presence of microsomes showed that this protein was not translocated and remained accessible on the cytoplasmic side (not shown). The signal sequence for E1 is present in the 6-kD protein (Melancon and Garoff, 1986). It was presumably only partially efficient in its function and produced a fraction of uncleaved and untranslocated E1 molecules with extra amino acids at the NH₂ terminus.

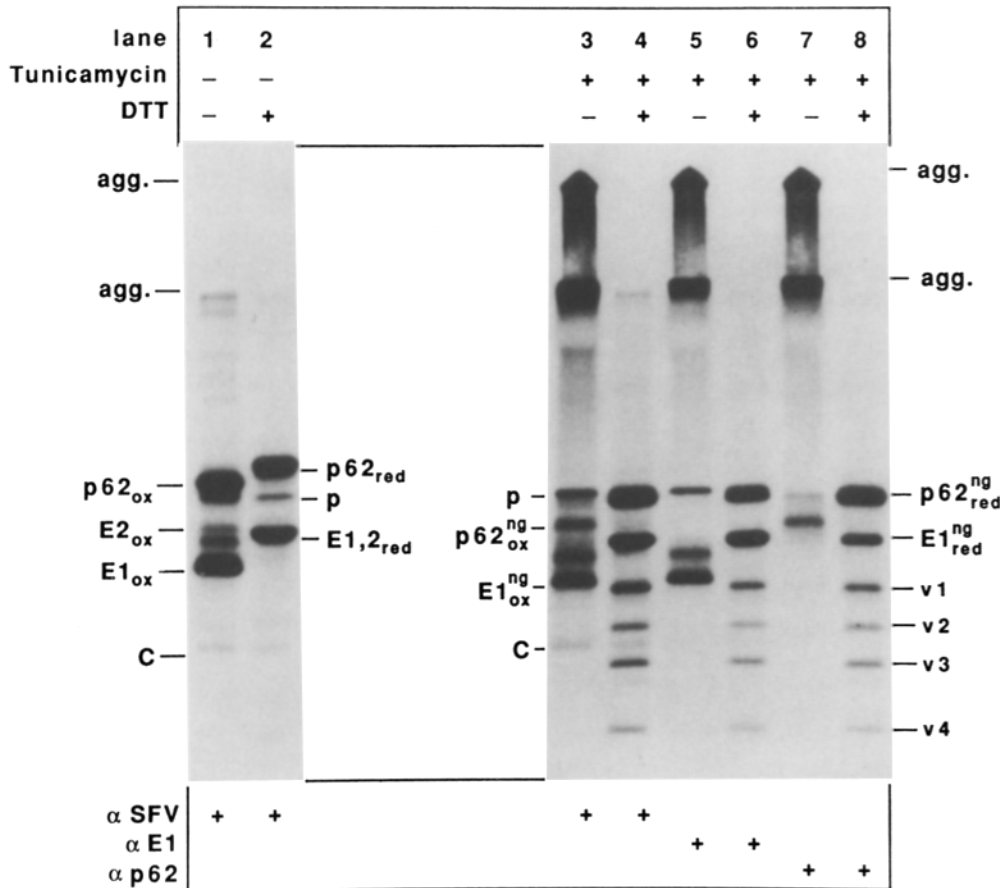When HeLa cells were cotransfected with both vectors, E1 and p62 were efficiently expressed. They showed the ex-

lane	1	2	3	4
DTT	−	+	−	+

p —

$E1_{red}$ —

$E1_{ox}$ —

— $p62_{red}$

— $p62_{ox}$
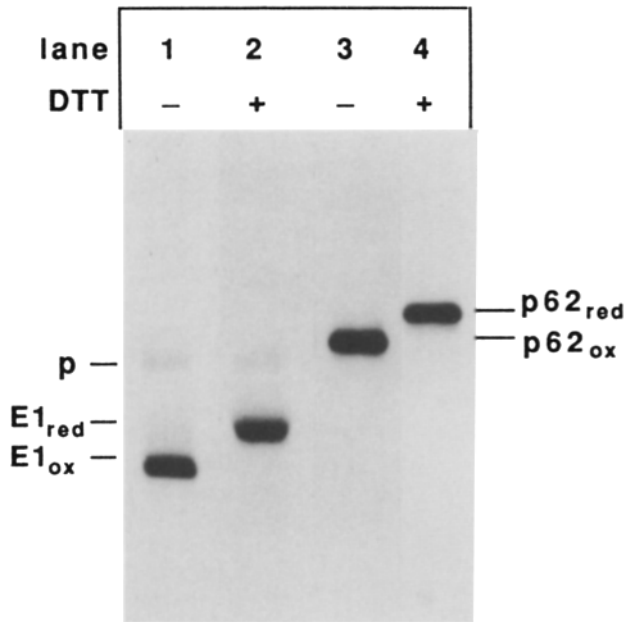
Figure 2. Expression of E1 and p62. HeLa cells were transfected for 2 h with the E1-(lane *1* and *2*) or p62-(lane *3* and *4*) expression vector, respectively. After methionine starvation, cells were pulsed for 5 min and chased for 10 min. Immunoprecipitation from the lysates was done using the polyclonal anti-SFV spike glycoprotein antibody. Reduced and nonreduced samples of the immunoprecipitates are shown. *p* designates an extra band derived from the E1 expression vector.
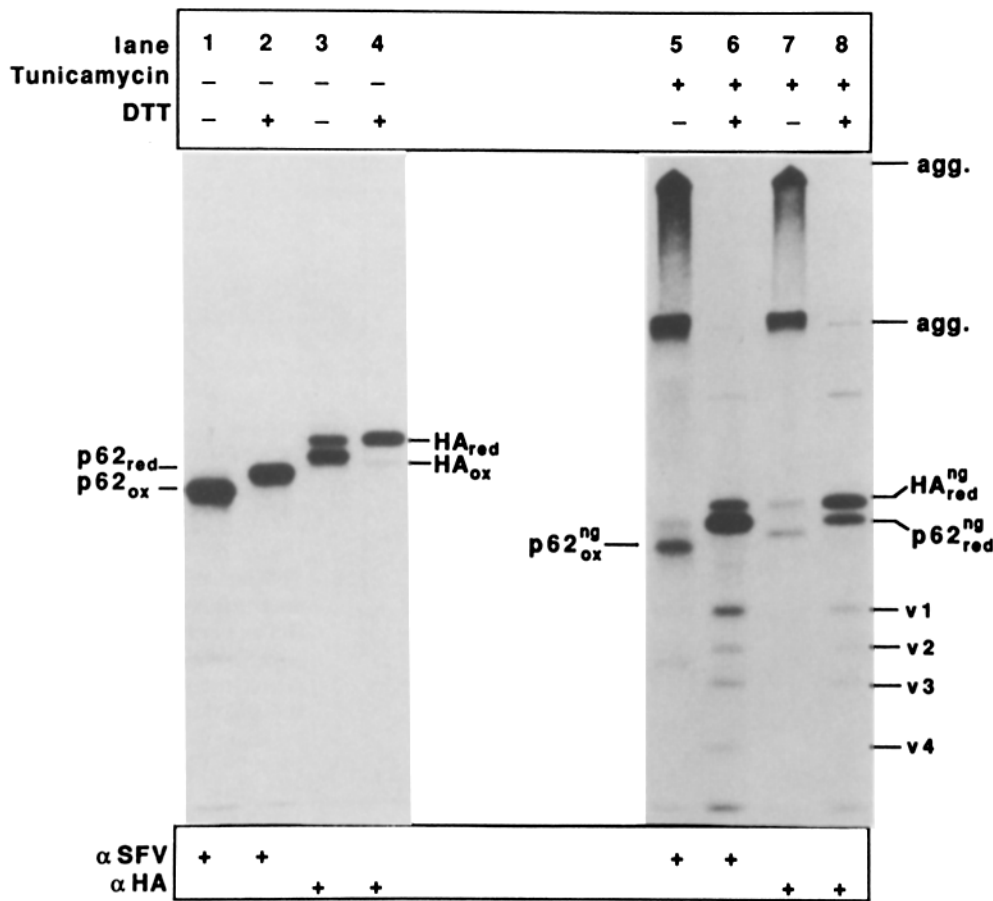
pected mobilities before and after reduction (Fig. 3, lanes *1* and *2*). When glycosylation was prevented by tunicamycin, most of the $p62_{ox}^{ng}$, and about half of the $E1_{ox}^{ng}$ were recovered in disulfide-linked aggregates at the top of the gel (lane *3*). Neither the cytosolic E1 form (*p*) nor the small amount of precipitated capsid protein (*C*) were part of the aggregates. Upon reduction the aggregates disappeared and prominent bands of nonglycosylated E1 and p62 could be seen (lane *4*). Precipitations with anti-E1 (lanes *5* and *6*) and anti-p62 (lanes *7* and *8*) antibodies showed that the disulfide cross-linked complexes were mixed. Clearly, coaggregation of nonglycosylated E1 and p62 occurred whether they were synthesized on the same or on different polysomes.

It is noteworthy that, in addition to E1 and p62, the reduced gels revealed the presence of four other labeled proteins in the aggregates. These are called v1, v2, v3, and v4 in Fig. 3 and subsequent figures. They were in the 20–50-kD range and did not represent SFV proteins. Since they could only be seen in aggregates obtained from the vaccinia virus-based expression system, they probably represented nonglycosylated, vaccinia-related proteins which had become part of the mixed aggregates. The molecular weights were consistent with some of the known glycoproteins of vaccinia (Carrasco and Bravo, 1986). Their presence in the disulfide crosslinked aggregates with E1 and p62 provided further evidence that the composition of the aggregates was mixed.

To confirm that aggregation was nonspecific, we coexpressed SFV p62 and influenza hemagglutinin. When immunoprecipitated from the double-transfected cell lysates.

lane	1	2		3	4	5	6	7	8
Tunicamycin	−	−		+	+	+	+	+	+
DTT	−	+		−	+	−	+	−	+

agg.—

agg.—

$p62_{ox}$ —

$E2_{ox}$ —

$E1_{ox}$ —

C —

— $p62_{red}$
— p
— $E1,2_{red}$

p —

$p62_{ox}^{ng}$ —

$E1_{ox}^{ng}$ —

C —

— agg.

— agg.

— $p62_{red}^{ng}$

— $E1_{red}^{ng}$

— v1

— v2

— v3

— v4

α SFV	+	+		+	+				
α E1						+	+		
α p62								+	+

Figure 3. Tunicamycin-induced aggregate formation in double-transfected cells. HeLa cells were double transfected with E1 and p62 for 1 h. After a 45-min preincubation with or without tunicamycin the cells were labeled and chased for 30 min each in the presence or absence of the drug. The same lysate was immunoprecipitated with the antibodies against SFV spike glycoproteins (*αSFV*), E1 (*αE1*), or p62 (*αp62*), respectively. *agg*, aggregates; *C*, capsid; *ng*, nonglycosylated; *p*, E1 precursor; *v1-4*, vaccinia-related proteins.

— agg.

— agg.

p62red—
p62ox —

—HAred
—HAox

HAng red

p62ng ox—

p62ng red

— v1
— v2
— v3
— v4

αSFV + +   + +
αHA   + +   + +

Figure 4. Tunicamycin-induced aggregate formation of coexpressed unrelated proteins. HeLa cells were double transfected with X31-HA and p62 for 1 h. After a 45-min preincubation in the presence or absence of tunicamycin the cells were labeled and chased for 30 min each. The same lysate was immunoprecipitated with the antibodies against SFV spike glycoproteins (αSFV), or influenza hemagglutinin (αHA), respectively. Lanes 1–4 show precipitations from untreated cells; lanes 5–8 equivalent samples from cells treated with tunicamycin.

influenza HA was present as a single band after reduction (Fig. 4, lane 4) and as a faster migrating doublet without reduction (lane 3). The upper band in the doublet corresponds to folding intermediates with incomplete disulfides and the lower band to fully oxidized, mature HA (Braakman et al., 1991). When synthesized in the presence of tunicamycin, most of the p62 and influenza HA formed disulfide-linked aggregates (lanes 5 and 7). Reduced precipitates with anti-SFV and anti-HA antibodies respectively (lanes 6 and 8), indicated that the aggregates were mixed. In this case, the aggregates contained proteins from three different sources: p62 from SFV, HA from influenza and the vaccinia-related proteins. This demonstrated that unrelated proteins form mixed heteroaggregates when their folding in the ER is perturbed.

Kinetics of Heteroaggregate Formation

HA aggregation was previously found to occur very soon after chain termination (Hurtley et al., 1989). Since our results indicated that aggregates can be composed of products from separate polysomes and not just locally from individual polysomes, it was of interest to determine how rapidly heteroaggregates were formed. It was possible that complexes between peptides from individual polysomes formed first and then coalesced to larger heterologous aggregates.

HeLa cells transfected with p62 and E1 were treated with tunicamycin, pulsed for 1 min, and chased for various times. Cycloheximide was added to the chase medium to block delayed synthesis of partially labeled nascent chains (see Braakman et al., 1991). At the end of the chase, the cells

were treated with NEM to block the formation of disulfide bonds, and the lysates were precipitated with the anti-p62 antibody. The immunoprecipitates were reduced and analyzed by SDS-PAGE. The p62 and the coprecipitated E1 bands were quantitated by densitometry (Fig. 5).

aggregated protein (% of total) vs chase (min)

p62

E1
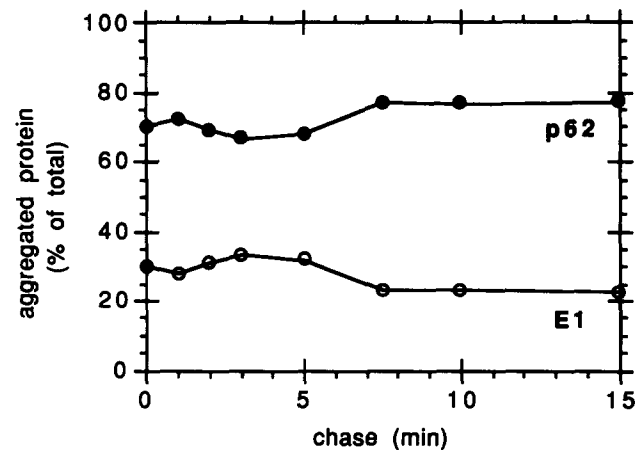
Figure 5. Kinetics of heteroaggregate formation. HeLa cells were preincubated with tunicamycin 2 h after the start of a double transfection with the E1- and p62- expression vectors. A pulse for 1 min in the presence of the drug was followed by a chase in the presence of cycloheximide for the times indicated. The lysates were precipitated with anti-p62. The immunoprecipitates were reduced, separated by SDS-PAGE, and analyzed by densitometry. The amounts of p62 and coprecipitated E1 are expressed as fraction of the total aggregated protein precipitated with the anti-p62 antibody.
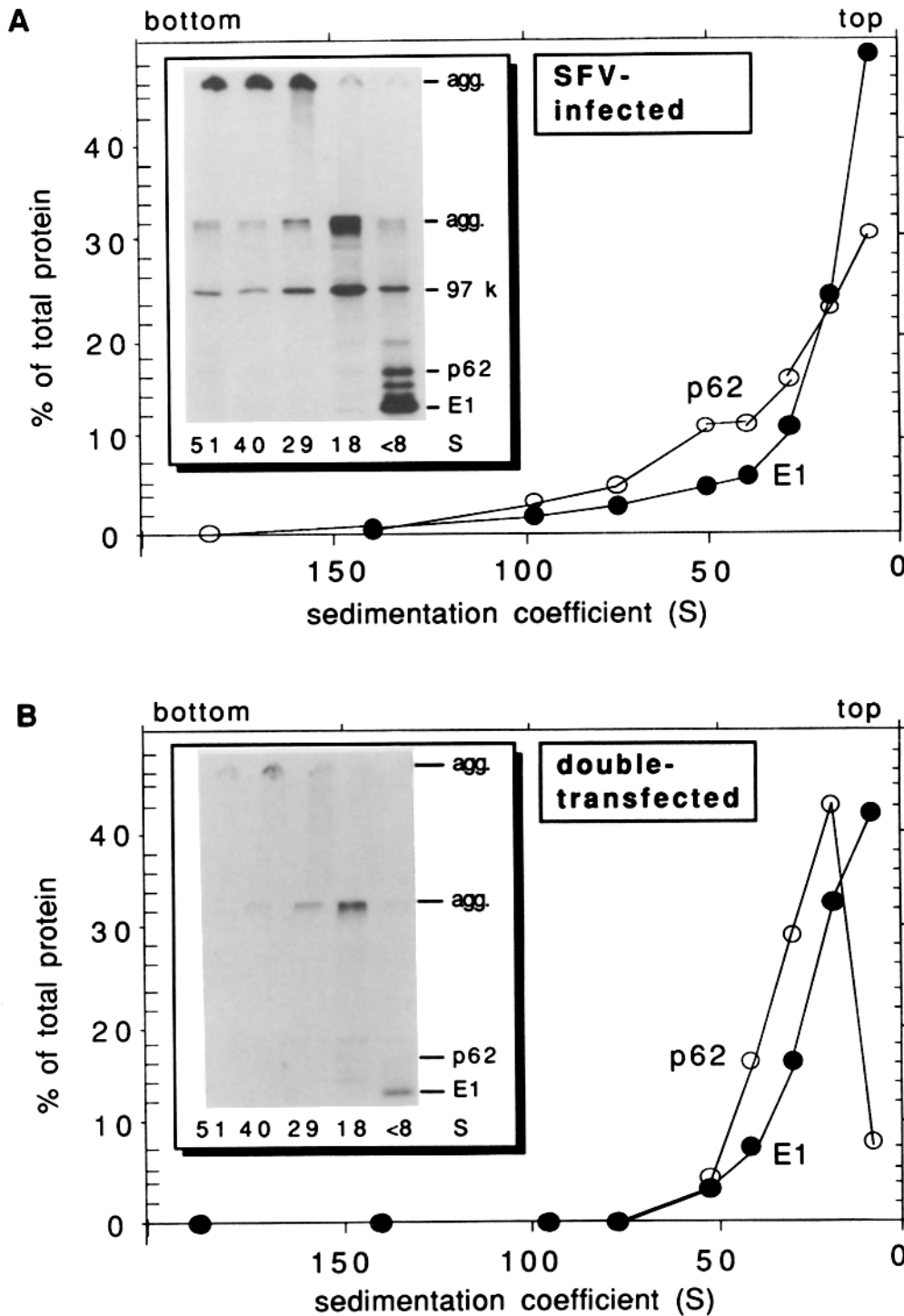
Figure 6. Aggregate size in SFV-infected cells (*A*) and in double-transfected cells (*B*). HeLa cells were either infected with SFV as described (*A*) or double-transfected with E1 and p62 for 2 h (*B*). In the presence of tunicamycin, a pulse for 30 min was followed by a chase for the same time. The lysate was loaded on top of a 15–30% sucrose gradient containing 0.5% Triton X-100 and spun for 40 or 150 min at 50,000 rpm (SW 50.1; Beckman Instruments, Inc., Palo Alto, CA). Catalase and SFV nucleocapsid were used as sedimentation markers to calculate the sedimentation rate of the proteins of interest. The fractions were immunoprecipitated and separated by SDS-PAGE. Reduced samples were analyzed by densitometry. Percentages are plotted as fractions of the total amount of protein loaded. The graphs represent a combination of the data from the 40- and 150-min centrifugations. The inserts in *A* and *B* show nonreduced samples from different gradient fractions.

The results showed that mixed aggregates formed during the 1-min pulse. Since it is believed that no cotranslational interaction of nascent chains can occur because of the spacing of ribosomes on the ER membrane (Hurtley and Helenius, 1989), aggregate formation must occur instantly after release from the ribosome. Moreover, the relative amount of labeled E1 and p62 did not change significantly with chase time (Fig. 5). We conclude that heteroaggregate formation occurs without initial homoaggregate formation immediately after chain termination. It is thus much faster than normal oligomerization which requires specific interac-

tions between folded subunits. Aggregate formation is probably so rapid because it involves nonspecific interactions between partially folded, poorly soluble polypeptide chains.

Aggregate Size

To obtain an estimate for the size of the heteroaggregates, cells transfected with E1 and p62 and cells infected with SFV were treated with tunicamycin, pulsed with [35S]methionine and chased for 30 min each, alkylated, and solubilized with Triton X-100. The lysates were then subjected to sucrose velocity gradient centrifugation in the presence of 0.5% Tri-
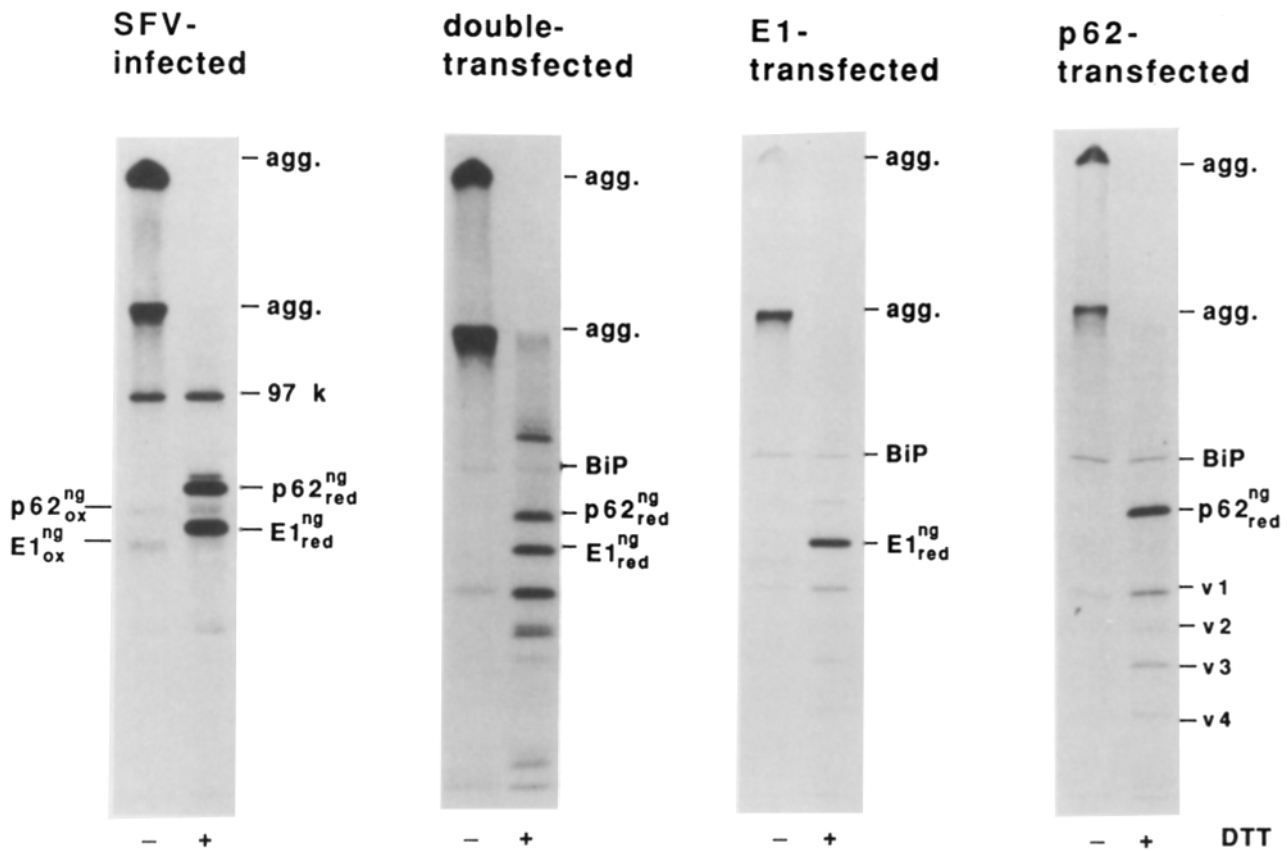
Figure 7. BiP/GRP78 association with tunicamycin-induced aggregates. HeLa cells were infected or transfected as described, preincubated with tunicamycin, and pulsed and chased for 30 min each. The cell lysates were prepared in the presence of N-ethylmaleimide and apyrase and kept at 0–4°C during the subsequent immunoprecipitations with the anti-BiP/GRP78 antibody. The samples were analyzed by SDS-PAGE in non-reduced and reduced form. Parallel immunoprecipitations with anti-SFV antibodies helped to identify coprecipitated SFV-proteins.

ton X-100. The distribution of viral proteins in gradient fraction was analyzed by immunoprecipitation and SDS-PAGE (Fig. 6).

In double-transfected cells the aggregate size was heterogeneous as indicated by the distribution of E1 and p62 in the gradient (Fig. 6 *B*). The sedimentation coefficients were in the 15–50 $S_{20,w}$ range suggesting molecular weights between 0.3 to 4 million. Most of the aggregates were low molecular weight species. The E1 protein that was not cross-linked to the aggregates (30–50% of total) was recovered on the top of the gradients as monomers (Fig. 6 *B, insert*). No change in size distribution was observed when the chase time was increased to 60 min (not shown). SFV-infected cells contained larger and more heterogeneous p62-E1 aggregates (Fig. 6 *A*). More than 10% of them sedimented with a coefficient between 50 and 200 $S_{20,w}$. The nonglycosylated proteins that had escaped interchain disulfide crosslinking were again recovered on the top of the gradient as monomers (Fig. 6 *A, insert*).

BiP/GRP78 Association to Unglycosylated Proteins

Misfolded protein aggregates in the ER are often associated with BiP/GRP78, an HSP70 homologue present in the ER lumen (see Hurtley et al., 1989). To determine whether this was true for SFV–protein complexes, we performed anti-BiP/GRP78 immunoprecipitations of lysates from tunicamycin-treated cells (Fig. 7). To stabilize the interaction of

BiP/GRP78 with its substrates, the cell lysates were depleted of ATP and kept at 0–4°C.

The anti-BiP/GRP78 antibodies efficiently precipitated the spike protein aggregates from SFV-infected cells, from cells transfected with either E1 or p62 alone, as well as from cells transfected with both E1 and p62 (Fig. 7). Evidently, binding to unglycosylated, aggregated proteins is a general property of BiP/GRP78. We noted, however, that BiP/GRP78 also associated with the nondisulfide linked and nonaggregated forms of E1 and p62 (see bands $E1_{ox}^{ng}$ and $p62_{ox}^{ng}$ in the non-reduced SFV-infected lysate, Fig. 7). Immunoprecipitations with nonspecific control antibodies showed that the 97-kD band seen in the anti-BiP precipitates represented an unspecific precipitation from the SFV-infected cell lysate (not shown).

Discussion

For most proteins that are cotranslationally translocated into the ER, folding begins on the nascent chains while they are still being translated and translocated (Bergman and Kuehl, 1979a,b; Tsou, 1988). When still associated with the polysome, some domains presumably reach their final conformation whereas folding in others is still not complete. During this time the polypeptide chains are fixed in place and unable to associate with each other and to form aggregates. The critical moment in terms of aggregation occurs immediately af-

ter chain termination when polypeptides are released from the polysome. Since some of their domains are only partially folded at this time, they are likely to be less soluble than mature, fully folded proteins. We think that the formation of mixed, random aggregates, as demonstrated here for viral proteins, is a consequence of the biophysical properties and the poor solubility of the folding intermediates, as extensively described in the literature on in vitro refolding (see Jaenicke, 1987).

A tendency of newly synthesized proteins to aggregate is observed in cells not only during formation of misfolded proteins but also during regular folding. There is increasing evidence that molecules that proceed to fold normally may undergo transient aggregation immediately after synthesis. Recent results with VSV G protein (Doms et al., 1987; de Silva, A., I. Braakman, and A. Helenius, manuscript in preparation) and thyroglobulin (Kim and Arvan, 1991) showed such complex formation. In the case of thyroglobulin, the newly synthesized proteins not only aggregated but also established transient interchain disulfide bonds similar to those seen as permanent features in misfolded protein aggregates. These examples demonstrate that, in the living cell, aggregates are not necessarily a kinetically irreversible trap for proteins failing to fold properly. They can represent an environment where coordinated folding can proceed, resulting in the final release and transport of the involved proteins. It is quite possible that numerous proteins undergo transient aggregation immediately after release from polysomes, and that the difference between a normally maturing and a misfolding protein is that the former continues to fold and dissociates from the aggregate, while the latter remains a permanent component of it.

We find that even without glycosylation inhibitors, a fraction of newly synthesized protein misfolds and aggregates in the ER (Fig. 1; Hurtley et al., 1989). The fraction of misfolded side products increases when cells are compromised by the vaccinia expression system, and it decreases when the temperature is lowered. Since identical polypeptide chains can end up in different final conformations, i.e., either as correctly folded monomeric species or as misfolded components of aggregates, we conclude that folding in the living cell is a stochastic process. This may explain the rather poor efficiency sometimes observed for protein maturation (see Hurtley and Helenius, 1989).

The inhibition of N-linked glycosylation increases the tendency for misfolding (Machamer and Rose, 1988b; Hurtley et al., 1989). Among the proteins analyzed here, the nonglycosylated p62 and HA were virtually quantitatively misfolded and aggregated. El molecules lacking the single N-linked sugar had an equal chance of ending up in an irreversible aggregate or escaping aggregation. The nonaggregated fraction of El had extensive intrachain disulfide bonds and was associated with BiP/GRP78. Since Garoff and co-workers have previously shown that El can only exit from the ER if it is associated with correctly folded p62 (Kondor-Koch et al., 1982), it is possible that the fraction of monomeric nonglycosylated El was correctly folded but trapped in the ER because there was no correctly folded p62 for heterodimerization.

When aggregation of newly synthesized proteins occurs, it is virtually instantaneous: the nonglycosylated p62 and El were aggregated and crosslinked by disulfide bonds within

1 min after chain termination. The complexes were mixed regardless of whether the proteins were derived from the same or from different polysomes. Several unrelated proteins could be present in the same aggregates, and all were covalently associated with each other. The only noncovalently attached component was BiP/GRP78, a resident ER chaperon commonly found associated with folding intermediates and misfolded proteins. The aggregate size ranged from small oligomers to large complexes with a molecular weight of several million daltons. The heterogeneous composition and size as well as the rapid kinetics of formation reinforced our interpretation that the process of aggregation constitutes a nonspecific precipitation reaction between poorly soluble folding intermediates in the plane of the ER membrane. Judging by our previous studies on influenza HA and tsO45 G protein from VSV, the aggregated proteins are not necessarily grossly unfolded, but may rather display conformational properties similar to normal folding intermediates (Hurtley et al., 1989; de Silva et al., 1990).

What is the role for N-linked glycosylation in protein folding? The N-linked sugars are often dispensable for the solubility and function of a mature folded protein, but critical during the initial folding of many proteins (Schülke and Schmidt, 1988; for review see Olden et al., 1982; Paulson, 1989). We think that one of the most important effects of the addition of preassembled oligosaccharide units to nascent chains is that it increases the overall solubility of the protein during posttranslational folding thus counteracting their tendency to form irreversible aggregates. In other words, the core sugars with the three terminal glucoses would, in addition to local effects on folding, have a global effect on the solubility properties. In vitro mutagenesis studies on VSV G protein have shown that N-linked carbohydrate side chains inserted into novel positions in the sequence can sometimes replace the normal sugar moieties and allow correct folding (Machamer and Rose, 1988a;b). This is consistent with the notion that the effect of N-linked sugars on folding is not merely local.

It is apparent that the efficiency of protein folding in the ER depends on a delicate balance of intra- and intermolecular interactions determined by the properties of the newly released protein, by the availability of folding enzymes and chaperones, and by the general conditions in the ER. The balance is tipped towards misfolding and permanent aggregation when glycosylation is inhibited, when the cells are compromised by virus infection, when the temperature is elevated, or, as we have recently shown for HA, when cells are depleted of ATP (Braakman et al., 1992). Aggregates are often associated with BiP and represent dynamic complexes which contain a spectrum of misfolded and partially folded proteins. Analyzing the nature of the aggregates further will hopefully allow a more complete picture of protein folding in the ER.

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