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Analysis In Vivo of GRP78-BiP/Substrate Interactions and Their Role in Induction of the *GRP78-BiP* Gene

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The endoplasmic reticulum (ER)-localized chaperone protein, GRP78-BiP, is involved in the folding and oligomerization of secreted and membrane proteins, including the simian virus 5 hemagglutinin-neuraminidase (HN) glycoprotein. To understand this interaction better, we have constructed a series of HN mutants in which specific portions of the extracytoplasmic domain have been deleted. Analysis of these mutant polypeptides expressed in CV-1 cells have indicated that GRP78-BiP binds to selective sequences in HN and that there exists more than a single site of interaction. Mutant polypeptides have been characterized that are competent and incompetent for association with GRP78-BiP. These mutants have been used to show that the induction of GRP78-BiP synthesis due to the presence of nonnative protein molecules in the ER is dependent on GRP78-BiP complex formation with its substrates. These studies have implications for the function of the GRP78-BiP protein and the mechanism by which the gene is regulated.

INTRODUCTION

The 78-kDa glucose-regulated/Ig heavy-chain binding protein (GRP78-BiP) is a member of the highly conserved 70-kDa heat shock (hsp70) family of stress-related proteins and is a soluble nonglycosylated protein found in the endoplasmic reticulum (ER) of all eukaryotic cells studied to date (Munro and Pelham, 1986; for reviews, see Pelham, 1989; Rothman, 1989; Gething and Sambrook, 1990). GRP78-BiP was identified independently by different groups through its increased expression in cells deprived of glucose (Pouysségur *et al.*, 1977) in lymphoid cells and by its association with Ig heavy chains that are not associated with Ig light chains (Haas and Wabl, 1983; Bole *et al.*, 1986). The polypeptides identified by these groups were later shown to be identical, thus linking its glucose-regulated expression with a biochemical property (Munro and Pelham, 1986). Other cellular perturbations that result in an induction of GRP78-BiP synthesis include inhibition of N-linked glycosylation and modification (Pouysségur *et al.*, 1977; Watowich and Morimoto, 1988), disruption of intracellular calcium stores (Drummond *et al.*, 1987), infection by enveloped viruses (Peluso *et al.*, 1978; Stoeckle *et al.*, 1988), the expression in the ER of mutant polypeptides deficient in folding (Kozutsumi *et al.*, 1988), and the increased expression of secretory proteins (Dorner *et al.*, 1989; Wiest *et al.*, 1990). These treatments affect processes in the ER where GRP78-BiP is localized, which suggests the existence of a feedback loop to regulate the expression of the gene.

Although the precise function of GRP78-BiP has not yet been defined, more recently it has been suggested to have a general role in the folding and assembly of proteins in the ER (Gething *et al.*, 1986; Munro and Pelham, 1986; Pelham, 1986), to mark aberrantly folded proteins destined for degradation (Gething *et al.*, 1986; Dorner *et al.*, 1987; Lodish, 1988; Hurtley *et al.*, 1989), or to aid in solubilizing aggregated proteins during times of stress (Munro and Pelham, 1986). Studies using viral membrane glycoproteins as models have implicated a role for GRP78-BiP in the general folding and assembly of proteins in the exocytic pathway. When the process of folding was monitored for the simian virus 5 (SV5) and Sendai virus hemagglutinin-neuraminidase (HN) proteins, as well as the vesicular stomatitis virus (VSV) glycoprotein (G), it was found the GRP78-BiP was bound transiently to these proteins until folding was complete (Ng *et al.*, 1989; Machamer *et al.*, 1990; Roux,

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1990). In a related study it was demonstrated that preexisting Ig heavy chains complexed with GRP78-BiP can be displaced by the in vivo addition of Ig light chains, resulting in the assembly and secretion of Ig (Hendershot, 1990). In addition to its role in assembly of HN and G, GRP78-BiP has been observed to be more stably associated with altered forms of HN or G that are malfolded (Machamer *et al.*, 1990; Ng *et al.*, 1990), as has also been found with malfolded forms of several other proteins, including the influenza virus hemagglutinin (Gething *et al.*, 1986; Hurtley *et al.*, 1989).

GRP78-BiP has been classified as a member of a family of proteins termed molecular chaperones (Ellis and Hemmingsen, 1989) or polypeptide chain-binding proteins (Rothman, 1989). This class of proteins also includes the GroEL/GroES and DnaK proteins of *Escherichia coli*, the subunit binding protein of plant chloroplasts, hsp60 of mitochondria, and the cytosolic forms of the hsp70 family. These proteins share the common characteristic of binding transiently newly synthesized proteins during the process of folding and assembly and thus have been implicated in guiding or promoting the folding and assembly of nascent proteins. Further evidence for these roles came from genetic studies in which it was found that yeast hsp60 is required for the assembly of several mitochondrial enzymes, as well as its own assembly (Cheng *et al.*, 1989, 1990). In addition, biochemical studies using purified proteins have shown that DnaK can stabilize RNA polymerase against heat inactivation and also promote disaggregation and refolding of heat inactivated RNA polymerase (Skowyra *et al.*, 1990).

Although the mechanism by which molecular chaperones exert their influence on protein folding and assembly is unknown, it has been suggested that these molecules bind to nascent polypeptides during synthesis to prevent unfavorable intra- or interchain interactions and permit the formation of complex structures at a kinetically favorable rate (Ellis and Hemmingsen, 1989; Rothman, 1989; Gething and Sambrook, 1990). A determination of the nature of the interaction between molecular chaperone proteins and their substrates is crucial to understanding the process. Flynn and coworkers (1989), by use of purified GRP78-BiP protein and a panel of synthetic peptides, showed that in vitro GRP78-BiP can bind to peptides as small as 10 amino acids and that their release was dependent on ATP hydrolysis by GRP78-BiP. In addition, binding affinities appeared to be variable and depended on the sequences of the peptides.

To understand the nature of the HN/GRP78-BiP interaction further, we have constructed a panel of HN deletion mutants to investigate the possibility that select regions of the HN polypeptide chain exist to which GRP78-BiP can bind in vivo. The data indicate that more than one binding site for GRP78-BiP binding exists in the HN polypeptide. Analysis of mutant HN molecules, for which GRP78-BiP interactions could be demonstrated and for those which it could not be found, correlates with the accumulation of the GRP78-BiP mRNA, suggesting that the induction of *GRP78-BiP* transcription is dependent on GRP78-BiP complex formation with its substrate in the ER.

MATERIALS AND METHODS

Cell Culture

The TC7 clone of CV-1 cells were grown and maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). In experiments involving recombinant SV40 virus-infected cells, the cells were maintained in DMEM supplemented with 2% FCS.

Antibodies

A rabbit polyclonal antiserum, raised against gel-purified sodium dodecyl sulfate (SDS)-denatured HN (anti-HN_{SDS}), recognizes all forms of HN as described previously (Ng *et al.*, 1990). A rat monoclonal antibody (anti-BiP), specific for the cellular heavy chain binding protein (BiP), was kindly provided by Dr. Linda Hendershot, St. Jude Children's Research Hospital, Memphis, TN (Bole *et al.*, 1986). A rabbit polyclonal antiserum prepared against chicken pyruvate kinase (anti-PK) that also recognizes simian PK was described previously (Hiebert and Lamb, 1988). A monoclonal antibody specific for the SV5 membrane (M) protein was kindly provided by Dr. Rick Randall, University of St. Andrews, Fife, Scotland, UK. All antisera described were titrated using an immunoprecipitation assay and were then used in all experiments under conditions of antibody excess.

Expression of HN Mutant Proteins and Metabolic Labeling of Cells

SV40-recombinant viruses that express the HN proteins and HN mutants (see below) were prepared as described (Sheshberadaran and Lamb, 1991). For virus infections, subconfluent monolayers of CV-1 cells were washed in phosphate-buffered saline (PBS) and infected with SV40-recombinant virus in DMEM for 2 h at 37°C. Cells were then rinsed with DMEM, and the medium was replaced with DMEM supplemented with 2% FCS and maintained at 37°C. Metabolic labeling was typically carried out 42–44 h postinfection (p.i.). Cells were rinsed twice in PBS and incubated with cysteine- and methionine-deficient DMEM (DMEM cys^-/met^-) for 30 min and then labeled using 50–100 μCi/ml Tran[^{35}S]-label (ICN, Irvine, CA) in DMEM cys^-/met^- and incubated at 37°C for the times indicated. In procedures requiring a pulse-label followed by further incubation (chase), the labeling medium was removed and replaced with chase medium (DMEM, 2 mM unlabeled L-cysteine and L-methionine and 2% FCS) warmed to 37°C. The chase period was terminated by washing cells in ice-cold PBS followed by lysis with the appropriate detergent buffer for immunoprecipitation (see below).

Immunoprecipitation, SDS-polyacrylamide gel electrophoresis (PAGE), and Quantitation of Autoradiograms

Immunoprecipitation of HN from labeled infected-cell lysates was performed essentially as described (Lamb *et al.*, 1978) unless indicated. SDS-PAGE was done as described (Lamb and Choppin, 1976). Autoradiograms were quantitated by laser scanning densitometry on variable exposures of each gel to assure being within the linear range of the film. Densitometry and integration were performed as described (Hiebert and Lamb, 1988).

Immunoprecipitations of HN/GRP78-BiP complexes were performed under ATP-depleting conditions. Radioactively labeled cells were washed once in ice-cold PBS and lysed in 50 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.4), 150 mM NaCl, and 1% Triton X-100 (TNT buffer) containing 10 mM glucose and 5 I.U. hexokinase (Calbiochem, La Jolla, CA). Immunoprecipitation was then carried out as described above except that immune complexes were washed in TNT buffer five times; all steps were performed at 0–4°C.

Endoglycosidase H Digestion

HN and HN mutant proteins were immunoprecipitated from infected-cell lysates as described above and then released from immune complexes by the addition of 15 μl 10 mM Tris (pH 7.4)/0.4% SDS and heating to 100°C. An equal volume of 0.1 M NaCitrate (pH 5.3) and 1 mU of Endo H (Boehringer Mannheim Biochemicals, Indianapolis, IN) was then added, and the samples were incubated for 24 h at 37°C. Endo H digestion was terminated by the addition of SDS-PAGE sample loading buffer and boiling.

Construction of HN Ectodomain Deletion and Duplication Mutants

HN deletion and duplication mutants were constructed by standard recombinant DNA techniques (Sambrook *et al.*, 1989) using plasmid pSV103HN (Paterson *et al.*, 1985). pSV103HN is a shuttle vector derived from pBR322 and SV40 containing the HN gene. In eukaryotic cells, SV40-recombinant virus stocks can be generated with the HN gene under the control of the SV40 later region promoter and polyadenylation signals as described previously (Paterson *et al.*, 1985). The C-terminal truncation mutants HNT_1, HNT_2, and HNT_3, which express HN molecules of 419, 281, and 167 residues, respectively, were created by introducing an oligonucleotide linker (5′-TTAGTTAACTAG-3′) (Hiebert and Lamb, 1988) that contains termination codons in all three reading frames at restriction endonuclease sites (*Bst*EII, nucleotide 1317, *Sma*I, nucleotide 898, and *Bam*HI, nucleotide 554) in the HN sequence of pSV103HN (numbering from Hiebert *et al.*, 1985).

Construction of the molecule pSV103Δ165-278 in which HN residues 165–278 were deleted was done by joining the *Bam*HI site (nucleotide 554) (modified by treatment with *E. coli* DNA polymerase Klenow fragment) to the *Sma* I site (nucleotide 898) using standard double ligation of DNA restriction fragments into the pSV103 vector. pSV103Δ165-278 encodes an HN deletion protein of 451 residues. Construction of the molecule pSV103Δ46-361 was done by joining the *Bgl* I site (nucleotide 206) (modified by treatment with bacteriophage T_4 DNA polymerase) to the *Bam*HI site (nucleotide 1208) (modified by treatment with *E. coli* DNA polymerase Klenow fragment) by ligation of DNA restriction fragments into the pSV103 vector. To obtain packaging into the SV40 capsid, the deletion was compensated by ligating a filler 594 bp *Bam*HI fragment, derived from the HN sequence, downstream of the HN translational termination codon. pSV103Δ49-361 encodes an HN deletion protein of 252 amino acids. $pSV103HNDT_3$ was constructed to extend the HNT_3 ectodomain. The HNT_3 ectodomain encoding region (residues 45–167 with the translational stop codon after residue 167) was fused to HN sequences at the *Bam*HI site (nucleotide 554) to create a protein of 281 residues containing four sites for the potential addition of N-linked carbohydrate. The junctions of all recombinant DNA constructions were verified by nucleotide sequencing.

Analysis of GRP78-BiP mRNA Abundance

Cytoplasmic RNA was isolated from virus-infected or mock-infected CV-1 cells as described previously (Collins *et al.*, 1978). Briefly, cell monolayers were washed with PBS and then lysed in buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 1% β-mercaptoethanol. The nuclei were removed by centrifugation, and the RNA-containing supernatant was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), followed by precipitation in ethanol. Nuclease S1 protection assays of endogenous GRP78-BiP mRNA were done similarly to those described by Wu and coworkers (1985), using a human GRP78-BiP gene probe (detailed in the legend to Figure 7). After overnight hybridization of the denatured probe to CV-1 cell RNA, single stranded nucleic acids were digested with 600 units of nuclease S1 (Boehringer Mannheim Biochemicals) for 60–90 min at 37°C. Proteins were extracted with phenol:chloroform:isoamyl alcohol and nucleic acids precipitated with ethanol. Nuclease S1-resistant products were separated by electrophoresis on 4% polyacrylamide gels containing 8 M urea and visualized by autoradiography.

RESULTS

Interaction of GRP78-BiP with HN Polypeptide Deletion Mutants

We have shown previously that HN, as well as HN mutant proteins that do not fold properly, are associated with GRP78-BiP in coimmunoprecipitation assays (Ng *et al.*, 1989, 1990). To determine whether the HN polypeptide contains a single site or more than one site for GRP78-BiP interaction, the cDNA clone of HN was altered, as described in MATERIALS AND METHODS, so that truncated and deleted HN proteins could be expressed in cells using an SV40-recombinant virus vector system. The truncation and deletion mutants of HN are shown schematically in Figure 1. The truncations and deletions can be considered random as we were only guided in their construction by the existence of convenient restriction sites in the HN cDNA. HN truncation mutants, HNT_1, HNT_2, and HNT_3, encode the HN N-terminal 419, 278, and 164 residues, respectively. No additional residues are found in HNT_1, but for HNT_2 and HNT_3, a Leu-Val-Asn tripeptide was added at their C-termini derived from the linker sequences containing the translational termination codons. SV40 recombinant virus stocks were prepared and diluted, so that expression of the mutant proteins in molar terms varied by less than a factor of two.

To demonstrate the coprecipitation of GRP78-BiP with the altered HN proteins, SV40 recombinant virus-infected CV1 cells were metabolically labeled at 42 h p.i. for 1 h with Tran[^{35}S]-label. Cells were lysed in detergent buffer and HN and HN mutant proteins were immunoprecipitated with polyclonal antiserum raised against SDS-polyacrylamide gel purified HN (anti-HN_{SDS}) (Ng *et al.*, 1990) and analyzed by SDS-PAGE. As shown in Figure 2, HN coprecipitates with a 78-kDa protein that we have previously identified as GRP78-BiP (lane HN/-ATP). As a control, parallel immunoprecipitations were performed in the presence of 1 mM ATP and in the absence of hexokinase. Consistent with previous observations (Munro and Pelham, 1986), GRP78-BiP specifically is dissociated from its substrates under these conditions (Figure 2, lane HN/+ATP). This is a useful control as with this antiserum, and the mild immunoprecipitation conditions used (to preserve the GRP78-BiP/substrate complex) a large number of non-

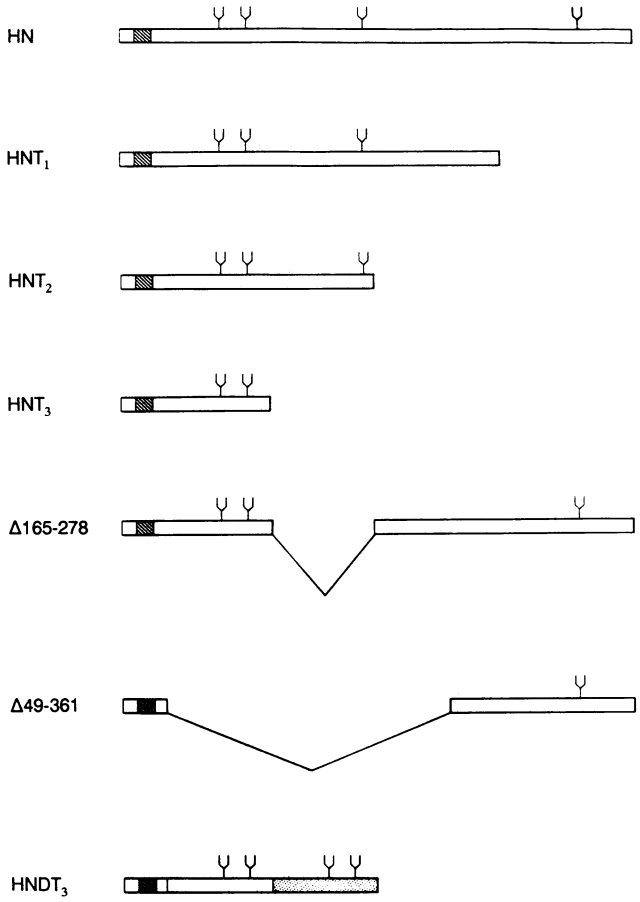

Figure 1. Schematic representation of HN deletion mutants. HN and HN mutant polypeptide amino acid sequences are represented by horizontal bars. The designated name for each polypeptide is indicated on the left. Transmembrane sequences are shown as a box shaded with diagonal lines, and potential sites of N-linked glycosylations that are used in HN (Ng *et al.*, 1990) are represented by the tree symbol. Sequences deleted from within the HN sequence are represented by intersecting diagonal lines. In $HNDT_3$, the duplication of the HNT_3 ectodomain (residues 49–164) is represented as the region in the blank horizontal bar followed by its duplicated sequence represented as a stipled bar.

specific species precipitated (including the SV40 polypeptide VP1 that comigrate with HNT_1) that are not found when more stringent conditions are used (see Figure 6C). When HNT_1 and HNT_2 were expressed, they also co-precipitated with GRP78-BiP, which suggests that sequences sufficient for a GRP78-BiP interaction exist in these truncated HN molecules (Figure 2). An apparent larger amount of labeled GRP78-BiP protein coprecipitates with HNT_1 and HNT_2 than with wild-type HN. This is not unexpected as we have previously shown that, whereas HN is transiently associated with GRP78-BiP, malfolded molecules of HN are stably associated with GRP78-BiP (Ng *et al.*, 1990). Given the nature of the deletions and the finding that none of the altered molecules are recognized by HN conformation-specific monoclonal antibodies (our unpublished data), it seems reasonable to assume the HN truncations result in the expression of non-native molecules. Interestingly, when HNT_3 was expressed, no detectable GRP78-BiP was coprecipitated (Figure 2), suggesting that the available sites for GRP78-BiP interaction with HN were deleted in HNT_3. However, we cannot rule out the possibility that a putative HNT_3/GRP78-BiP interaction exists that is not sufficiently stable to be detected in the coimmunoprecipitation assay. It is clear that any association of GRP78-BiP with HNT_3 is not of the type observed with the HN, HNT_1, or HNT_2 molecules.

The difference between HNT_2 and HNT_3 in their detectable association with GRP78-BiP suggests that a site for interaction is located within the sequences that differ between the two proteins. To investigate if a single GRP78-BiP interaction site is located in HN between residues 165–278, the HN deletion mutant Δ165–278 was constructed (Figure 1). When Δ165–278 was expressed, it was found to coprecipitate with GRP78-BiP (Figure 3), which suggests that HN contains more than one site for GRP78-BiP interactions. To explore this possibility further, a more extensive deletion mutant (Δ49–361) was constructed in which the C-terminal 204 amino acids were brought closer to the transmembrane domain, thus essentially eliminating the HNT_2 ectodomain sequences from HN. When Δ49–261 was expressed, it was found to coprecipitate with GRP78-BiP (Figure 3). Thus, taken together, these data indicate that the HN polypeptide chain contains more than one site for interaction with GRP78-BiP.

Although GRP78-BiP can bind short peptides in vitro (Flynn *et al.*, 1989), it was thought possible that the lack of a detectable association between HNT_3 and GRP78-BiP in vivo was because the HNT_3 polypeptide was too small (167 residues) to support the type of interaction found with the other HN derivative molecules. To address this question, a mutant molecule was constructed to lengthen the HNT_3 polypeptide by duplicating its C-terminal 114 amino acids. The new protein, designated $HNDT_3$, contains 281 amino acids (HNT_2 is also a 281 amino acid protein) and four consensus sites for the potential addition of N-linked carbohydrates (see Figure 1). As shown in Figure 3, when $HNDT_3$ was expressed and immunoprecipitated, a small but detectable amount of GRP78-BiP was coprecipitated (compare + and − ATP lanes). If the association of GRP78-BiP with substrate molecules depended on polypeptide length, $HNDT_3$ should coprecipitate a similar or greater amount of GRP78-BiP than the shorter Δ49–361. However, as shown in Figure 3, the Δ49–361 polypeptide, which consists of 29 fewer amino acids and three fewer N-linked carbohydrate chains than $HNDT_3$, coprecipitated a far larger amount of GRP78-BiP than $HNDT_3$ (Figure 3, cf. lane Δ49–361/−ATP to lane $HNDT_3$/−ATP), which suggests that polypeptide sequence rather than length is responsible for the observed GRP78-BiP in-

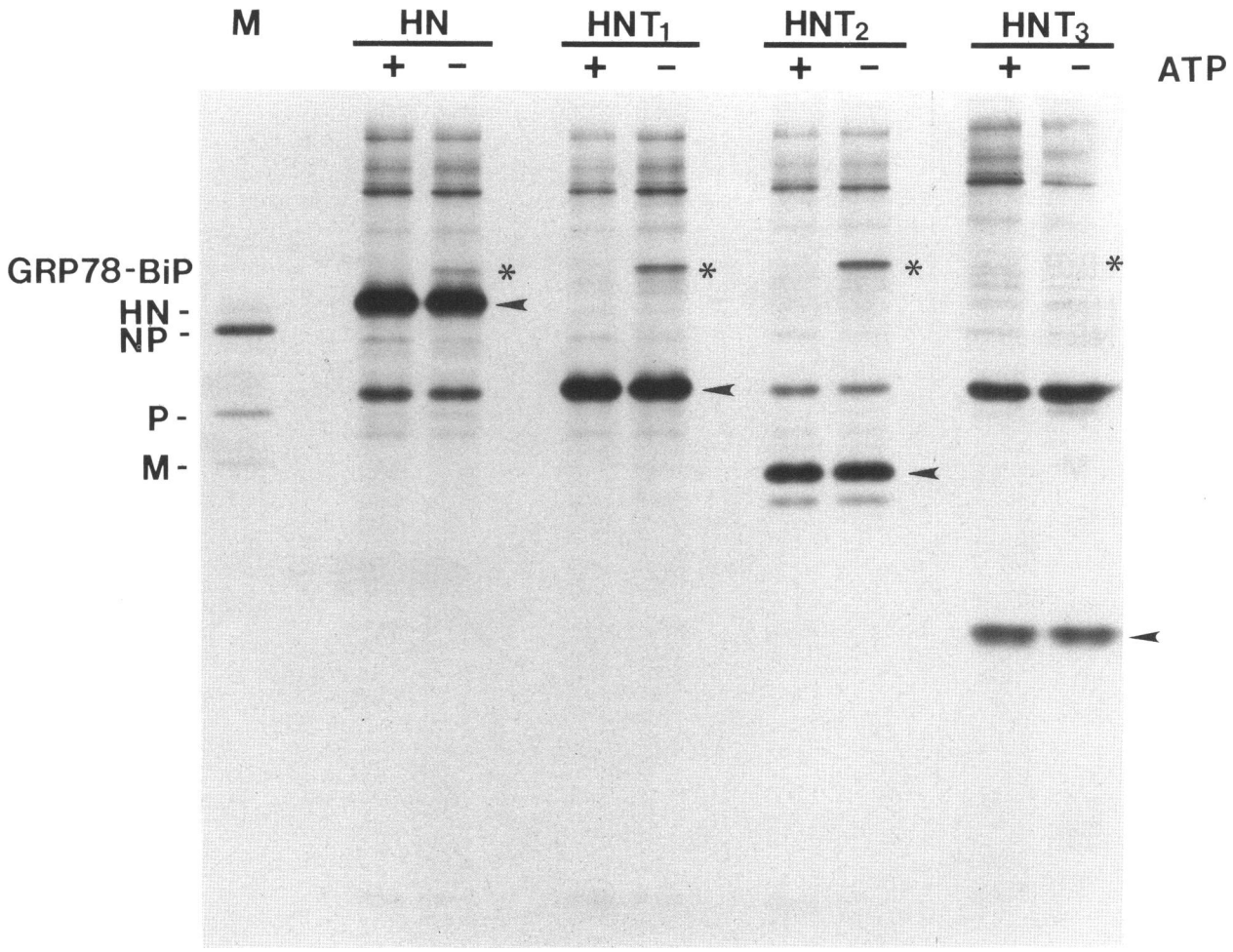

Figure 2. GRP78-BiP is associated with some but not all HN truncation mutants. SV40 recombinant virus-infected CV-1 cells in duplicate dishes were metabolically labeled at 44 h p.i. with Tran[^{35}S]-label for 1 h. The cells were then rinsed in PBS at 4°C and lysed in lysis buffer containing either D-glucose and hexokinase or ATP. HN and HN mutant proteins were immunoprecipitated with anti-HN_{SDS} serum and analyzed by SDS-PAGE and fluorography. Lane M, SV5-infected cell marker polypeptides: HN, 66kDa; NP, 61kDa; P, 44kDa; M, 38kDa. Arrowheads mark positions of HN and HN mutant proteins. *, Position of GRP78-BiP. +, Cells lysed in detergent buffer containing 1 mM ATP in detergent buffer. −, Cells lysed in the presence of 10 mM D-glucose and 5 I.U. hexokinase.

teraction. The small amount of GRP78-BiP that was coprecipitated with $HNDT_3$ may be due to the novel junction created during the construction of $HNDT_3$, providing a weak GRP78-BiP recognition site not present in HNT_3.

It was thought possible that the inability to detect a considerable association of GRP78-BiP with mutant proteins HNT_3 and $HNDT_3$ could be due to a rapid transient association of GRP78-BiP with the molecules. To investigate this possibility, pulse-chase experiments were performed. SV40 recombinant virus-infected cells expressing HN, HNT_2, HNT_3, and $HNDT_3$ were metabolically labeled for 15 min with Tran[^{35}S]-label and then incubated in chase medium for 0 or 90 min. The cells were lysed with detergent buffer under ATP-depleting conditions and aliquots were immunoprecipitated with anti-HN_{SDS} (Figure 4, lanes 1, 4, 7, and 10) or with anti-BiP monoclonal antibody (Figure 4, lanes 2, 3, 5, 6, 8, 9, 11, and 12). In cells expressing HN, the BiP antibody coprecipitated GRP78-BiP and HN, but after a chase period (lane 2), only a small amount of HN coprecipitated with GRP78-BiP (lane 3) indicative of the transient association of GRP78-BiP with maturing HN (Ng *et al.*, 1989). It should also be noted that many cellular polypeptides coprecipitated by the BiP-antibody also showed a transient association with GRP78-BiP, as they were detected after the pulse-label but not after the chase period (Figure 4, cf. lanes 2 and 3). The prominent band with a slightly slower mobility than the SV5 P protein marker is the SV40 VP1 protein that nonspecifically associated with protein A Sepharose-immunocomplexes under the mild immunoprecipitation

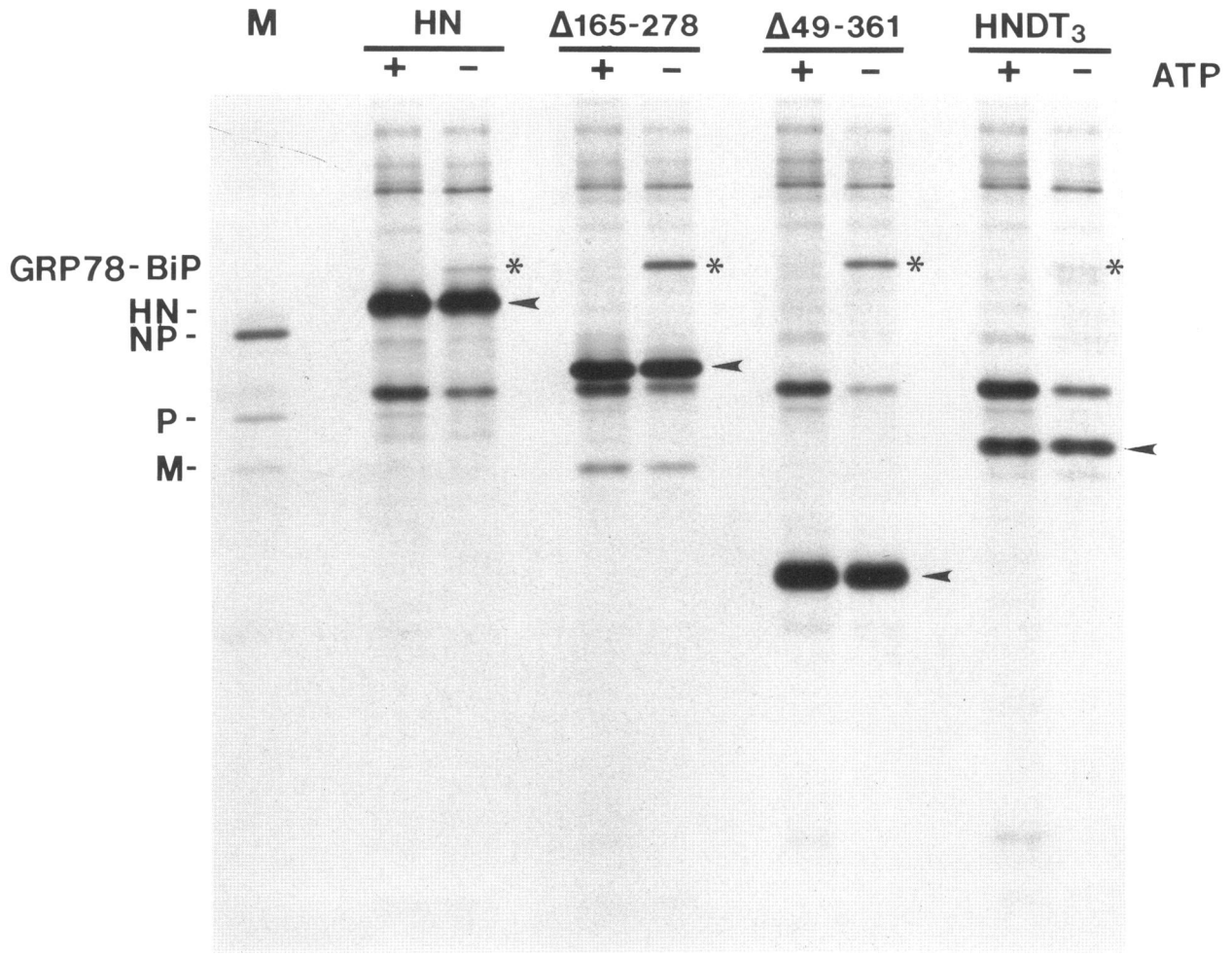

Figure 3. Association of GRP78-BiP with HN deletion and duplication mutants. SV40 recombinant virus-infected CV-1 cells in duplicate dishes were metabolically labeled at 44 h p.i. with Tran[^{35}S]-label for 1 h. The cells were then processed for immunoprecipitation as described in Figure 2 and analyzed by SDS-PAGE and fluorography. M, SV5 viral protein markers as in Figure 2. Arrowheads mark positions of HN and mutants proteins. *, Position of GRP78-BiP. +, Cells lysed in detergent buffer containing 1 mM ATP. −, Cells lysed in detergent buffer containing 10 mM D-glucose and 5 I.U. hexokinase.

conditions used (our unpublished data). When the same experiment was performed on cells expressing the HNT_2 protein, the BiP antibody coprecipitated the same amount of HNT_2 after the pulse-label and chase periods (Figure 4, cf. lanes 5 and 6), as expected for a malfolded HN molecule (Ng *et al.*, 1990). Unexpectedly, only a small portion of labeled HNT_2 molecules could be coprecipitated with GRP78-BiP using the BiP antibody (Figure 4, cf. lanes 5 and 6) in comparison to approximately half of the newly synthesized HN molecules coprecipitated with GRP78-BiP (Figure 4, cf. lanes 1 and 2). It does not seem likely that the amount of HNT_2 coprecipitated by the BiP antibody is representative of the amount of HNT_2 associated with GRP78-BiP in cells because a greater amount of GRP78-BiP is associated with the total population of HNT_2 than is associated with HN (Figure 4, cf. lanes 1 and 4). It is known that the stability of GRP78-BiP/protein complexes is sensitive to experimental conditions and dissociates in time after solubilization of cells (Hendershot and Kearney, 1988; Hurtley *et al.*, 1989). Thus, the difference can be explained if a greater number of GRP78-BiP molecules are associated with immature HN monomers than are associated with HNT_2 as the above data suggests. After solubilization of the cells, the instability of the GRP78-BiP/substrate complex will favor the recovery of those substrates initially associated with the most molecules of GRP78-BiP. To add credence to such an argument, it should be noted that when immature molecules of wild-type HN were isolated, only an average of 0.7 molecules of GRP78-BiP were detected for every HN monomer after solubilization of the cells (Ng *et al.*, 1989), yet the data shown here indicates there are a minimum of two sites for GRP78-BiP interaction with

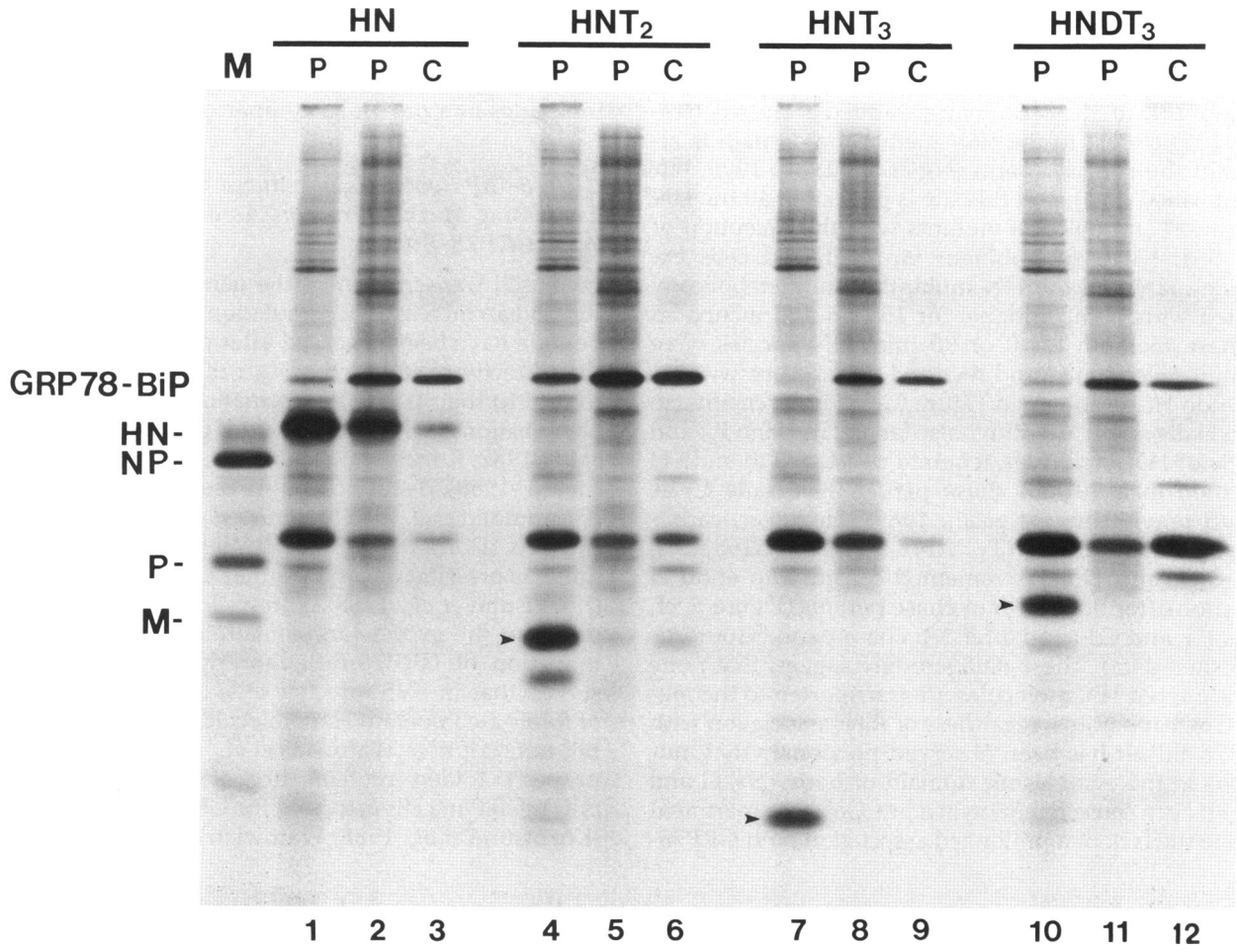

Figure 4. Pulse-chase analysis of GRP78-BiP association with HN mutant proteins. SV40 recombinant virus-infected CV-1 cells at 44 h p.i. were metabolically pulse-labeled with Tran[^{35}S]-label for 15 min and incubated in chase medium for 0 or 90 min. Detergent lysates were prepared under ATP-depleting conditions and aliquots immunoprecipitated with either anti-HN_{SDS} serum (lanes 1, 4, 7, and 10) or anti-BiP antibody (lanes 2, 3, 5, 6, 8, 9, 11, and 12). Radiolabeled polypeptides were analyzed by SDS-PAGE and fluorography. M, SV5 viral protein markers as in Figure 2. The position of GRP78-BiP is indicated to the left of the gel. P, pulse-label; C, pulse-label and 90 min chase. Arrowheads mark the positions of mutant polypeptides.

HN. Alternatively, GRP78-BiP/HNT_2 association may be weaker than GRP78-BiP/HN association and less able to withstand the experimental conditions.

When cells expressing HNT_3 and $HNDT_3$ were immunoprecipitated with the BiP antibody after a pulse-label or pulse-label and chase period, the HNT_3 and $HNDT_3$ polypeptides could not be detected even on prolonged exposures of autoradiographs (Figure 4, lanes 8, 9, 11, and 12). Thus, these data are consistent with the data shown in Figures 2 and 3, indicating that these molecules have no or slight association with GRP78-BiP and that within the limits of the assay the lack of detectable association is not due to a transient association.

Intracellular Localization of HN Mutant Proteins

Considerable evidence has accumulated indicating that for a protein to be transported out of the ER it must be properly folded and oligomerized (Gething *et al.*, 1986; reviewed in Hurtley and Helenius, 1989). As GRP78-BiP has often been found associated in a semistable manner with incompletely assembled or malfolded proteins, it has been proposed that it may play a role in retention of proteins in the ER (Bole *et al.*, 1986; Dorner *et al.*, 1987; Hurtley *et al.*, 1989). Therefore, it was of interest to investigate the fate of the HN truncation and deletion mutants in the exocytic pathways given their differing association with GRP78-BiP.

The anti-HN_{SDS} serum that recognizes all forms of HN was found to be unsuitable for staining of cells by immunofluorescence. However, the acquisition of carbohydrate chains resistant to digestion with endo-β-glycosidase H (endo H) is indicative of transport of glycoproteins to the medial Golgi complex (Kornfeld and Kornfeld, 1985). It has been shown previously that some of the four N-linked carbohydrate chains on HN are

modified from high mannose (endo H sensitive) to complex (endo H resistant) carbohydrate chains during intracellular transport. On HN molecules expressed at the cell surface, the carbohydrate added at the site (residue 110) closest to the transmembrane domain is always in the complex form (Ng *et al.*, 1989, 1990; our unpublished data), and this site is present in all the HN truncation and deletion mutants with the exception of Δ49–361. SV40 recombinant virus-infected cells expressing HN and the HN mutants were metabolically labeled with Tran[^{35}S]-label for 15 min and incubated in chase medium for 0 or 90 min. HN species were immunoprecipitated and digested with (+) or without (−) endo H. As shown in Figure 5, HN was sensitive to endo H digestion after the pulse label (HN, lane P), and >50% of HN molecules acquired resistance to endo H digestion in a 90-min chase period (HN, lane C) as shown previously (Ng *et al.*, 1989). However, carbohydrate chains on HNT_1, HNT_2, HNT_3, Δ165–278, Δ49–361, and $HNDT_3$ remained sensitive to endo H digestion after both 90-min chase periods (Figure 5, cf. lanes P+ and C+) and after 3-h chase periods (our unpublished data). These data strongly suggest that none of the altered HN molecules are transported to the medial Golgi apparatus, regardless of their association with GRP78-BiP. It has been observed previously that mutations in the cytoplasmic domain of both VSV G and SV5 HN can cause retention in a pre-Golgi compartment despite the lack of a prolonged association with GRP78-BiP (Doms *et al.*, 1988; Machamer *et al.*, 1990; Parks and Lamb, 1990). Thus, other signals/mechanisms must exist that positively or negatively cause retention of molecules in a pre-Golgi compartment.

GRP78-BiP Synthesis is Induced Only in Cells Expressing Mutant Proteins Associated With GRP78-BiP

Increased transcription of the gene encoding GRP78-BiP and an increased accumulation of GRP78-BiP polypeptide have been observed after perturbations to cells that directly or indirectly affect the ability of proteins to fold to their native conformations in the ER. These perturbations include inhibition of glycosylation (Chang *et al.*, 1987; Kozutsumi *et al.*, 1988; Watowich and Morimoto, 1988), disruption of intracellular calcium stores (Drummond *et al.*, 1987), increased expression of soluble proteins (Dorner *et al.*, 1989; Wiest *et al.*, 1990), and direct expression of proteins that cannot fold normally (Kozutsumi *et al.*, 1988; Watowich *et al.*, 1991). In addition to the expression of malfolded HN causing an induction of GRP78-BiP transcription, we have observed that in SV5-infected cells the flux of synthesis of folding-competent HN molecules stimulates GRP78-BiP transcription (Watowich *et al.*, 1991). Although improperly folded proteins are often associated with GRP78-BiP in cells activated for GRP78-BiP expression (Kozutsumi *et al.*, 1988; Watowich *et al.*, 1991), it is not

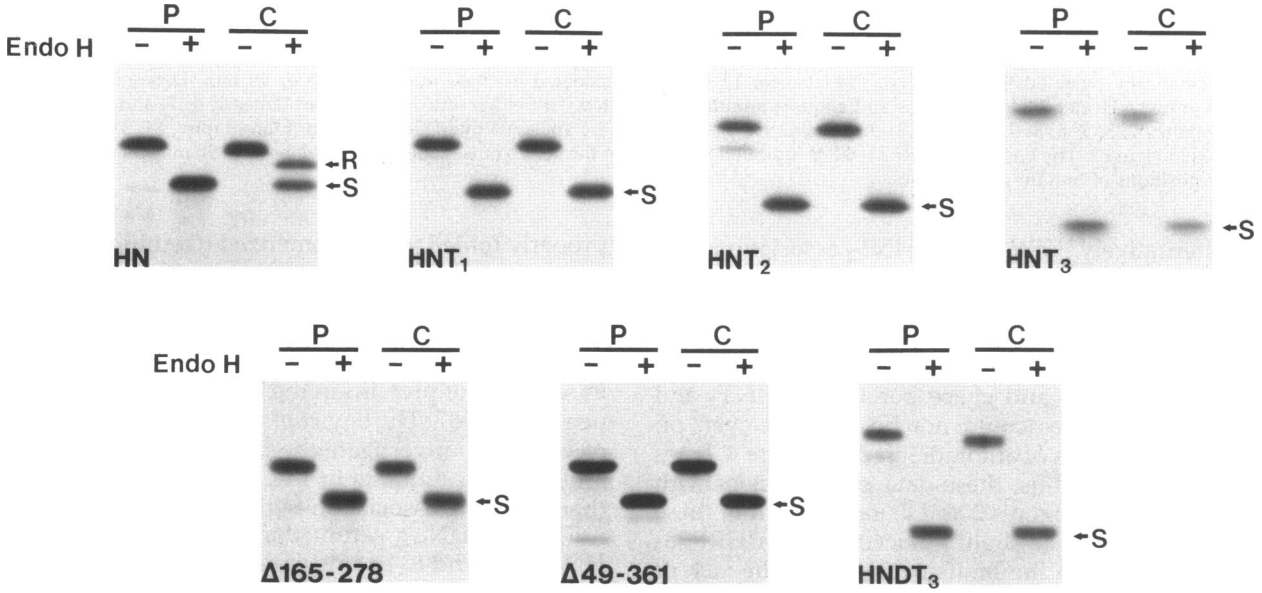

Figure 5. Susceptibility of HN mutant polypeptides to digestion by endoglycosidase H. SV40 recombinant virus-infected CV-1 cells at 42 h p.i. were pulse-labeled for 15 min with Tran[^{35}S]-label and incubated in chase medium for 0 or 90 min. HN and mutant polypeptides were immunoprecipitated as described (Lamb *et al.*, 1978) using anti-HN_{SDS} serum. Immunoprecipitated polypeptides were released from protein A-Sepharose beads by boiling in buffer containing 10 mM Tris (pH 7.4) and 0.4% SDS. An equal volume of 0.1 M NaCitrate (pH 5.3) was added, followed by 1 mU of endo H, and samples incubated at 37°C for 24 h. Polypeptides were analyzed by SDS-PAGE and fluorography. Only the relevant portion of the fluorograms are shown.

known whether binding is a prerequisite to induction. Insight into this question would contribute to the understanding of the signaling pathway from the ER to the nucleus and the HN deletion, and insertion mutants that do and do not associate with GRP78-BiP provide a useful experimental system.

Cells infected with SV40 recombinant-viruses expressing HN and HN mutants were labeled with [^{35}S]methionine for 1 h at 42 h p.i., detergent lysates were prepared, and aliquots were immunoprecipitated with anti-pyruvate kinase serum (to monitor for recovery of a cellular protein whose regulation is independent of GRP78-BiP), anti-BiP, or anti-HN_{SDS} serum (and a M MAb for control cells expressing the SV5-cytoplasmic M protein). As reported previously (Watowich *et al.*, 1991), SV40 mock infection or infection of cells with an SV40-recombinant virus expressing the SV5 cytoplasmic protein M did not cause an increase in GRP78-BiP synthesis, and HN synthesis caused a twofold increase in GRP78-BiP synthesis (Figure 6). However, in cells expressing HNT_1, HNT_2, Δ165–278 or Δ49–361, a 3–6 fold increase in GRP78-BiP synthesis was observed whereas for HNT_3 and $HNDT_3$ which did not form a detectable association with GRP78-BiP an increase in GRP78-BiP synthesis was not observed.

To determine the relative levels of GRP78-BiP mRNA accumulation in cells expressing HN truncation mutants, cytoplasmic RNA was prepared from cells at 24, 36, 42, and 48 h p.i. and analyzed by nuclease S1 protection. It has been shown previously using the SV40-recombinant virus vector system to express malfolded proteins that as the rate of recombinant protein synthesis increases between 24–48 h p.i., there is a concomitant increase in GRP78-BiP synthesis (Kozutsumi *et al.*, 1988; Watowich *et al.*, 1991). As shown in Figure 7, the accumulation of GRP78-BiP mRNA in mock-infected cells remained relatively constant during the time course. As expected, expression of HNT_2 caused a significant (8-fold) increase in levels of GRP78-BiP mRNA accumulation from 24 to 48 h p.i. However, a striking increase in GRP78-BiP mRNA accumulation was not observed in cells expressing HNT_3 or $HNDT_3$ over that found in mock-infected cells. A slight increase in mRNA accumulation in cells expressing $HNDT_3$ could be detected, but its significance is not clear as a corresponding increase in GRP78-BiP protein synthesis was not observed (see Figure 6D). Thus, taken together, these data suggest that only proteins that form an association with GRP78-BiP lead to induction of *GRP78-BiP* transcription and that the signaling pathway for *GRP78-BiP* activation involves the binding of GRP78-BiP to a substrate.

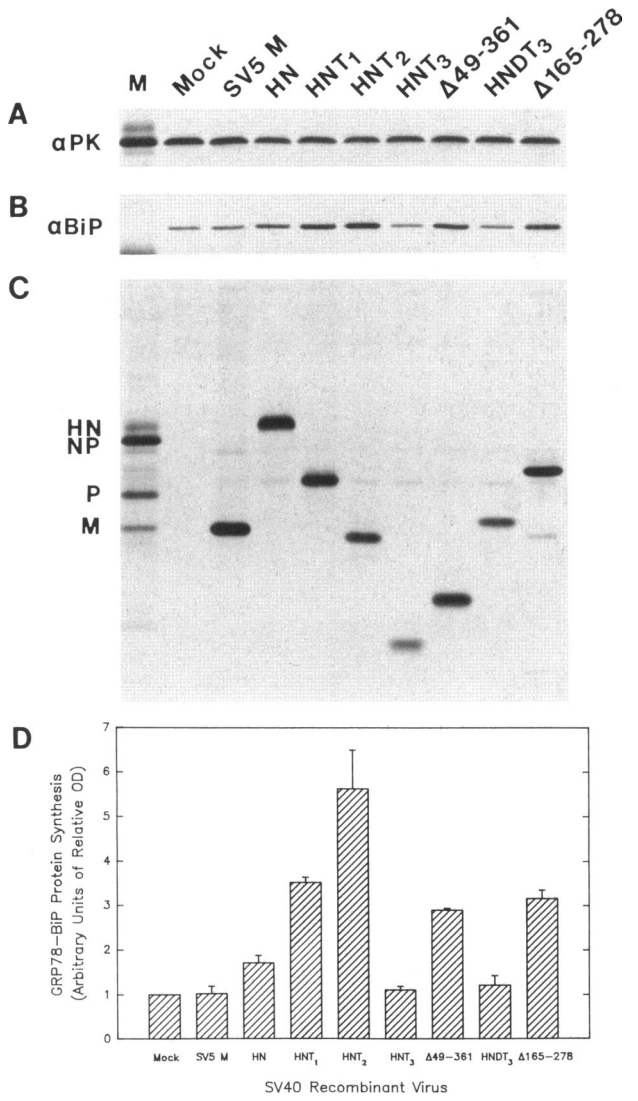

Figure 6. Differential induction of GRP78-BiP protein synthesis in cells expressing HN mutant proteins. SV40 recombinant virus-infected or mock-infected CV-1 cells at 42 h p.i. were metabolically labeled with [^{35}S]methionine for 1 h. Cells were lysed in detergent buffer (noncoprecipitation conditions) and aliquots were immunoprecipitated using anti-PK serum, anti-HN_{SDS} serum, (or anti-M mAb in the case of cells expressing SV5 M protein), or anti-BiP polypeptides were analyzed by SDS-PAGE and fluorography. Only the relevant parts of each fluorogram are shown. (A) Expression of pyruvate kinase. (B) Expression of GRP78-BiP. (C) Expression of recombinant proteins. (D) Quantitation of GRP78-BiP expression by laser scanning densitometry of fluorograms. Shown are the means of two independent experiments. Units of optical density obtained from densitometric scans are arbitrary with the levels of mock-infected cells set to 1. Error bars indicate the SEM.

DISCUSSION

Recently, it has become clear that many, if not most, proteins require the aid of molecular chaperones to attain their native structures and that these protein factors are found in all subcellular compartments where protein folding occurs (reviewed in Ellis and Hemmingsen, 1989; Rothman, 1989). However, the mechanism by which these chaperone molecules exert their effects is unclear. For example, it is unlikely that chaperone pro-

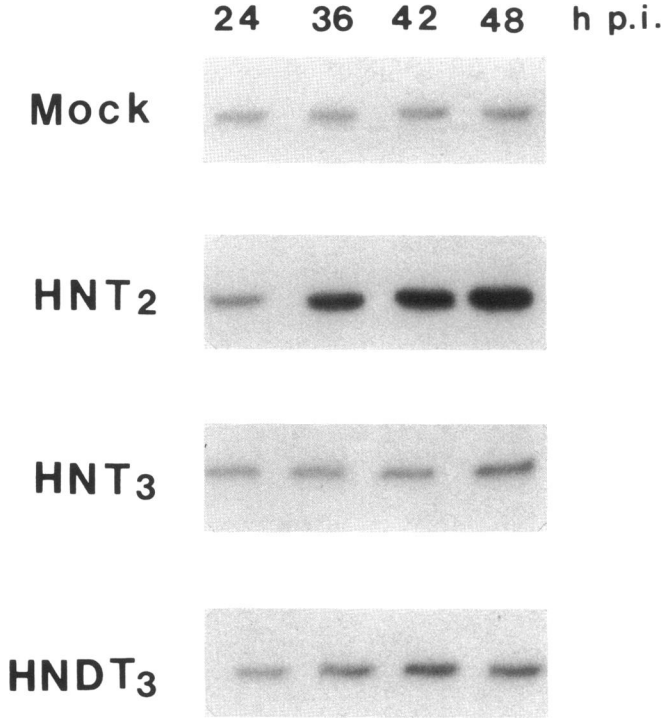

Figure 7. Timecourse of GRP78-BiP mRNA induction in cells expressing HN mutant polypeptides. CV-1 cells were infected with SV40 recombinant viruses expressing the HN mutants. Parallel cultures were mock-infected. At the times (h p.i.) indicated cytoplasmic RNA was isolated. The RNA was hybridized to a 650 bp *Bst*EII/*Pvu* II fragment, [^{32}P]-end-labeled at the *Bst*EII site, isolated from plasmid pHG26.8. This plasmid contains ~800 bp of 5′ sequences from the human GRP78-BiP cDNA (Watowich and Morimoto, unpublished results). Human GRP78-BiP RNA protects a 500-bp fragment of this probe, and the human cDNA is sufficiently homologous to simian mRNA, such that it can be used as a probe for RNA from CV-1 and it protects a fragment of identical size. Protected fragments were analyzed on 4% polyacrylamide gels containing 8 M urea and autoradiography and the region of the protected fragments are shown.

teins impart any structural information to substrate molecules because they bind a large variety of proteins. Also, chaperone proteins are not known to catalyze any post-translational covalent modifications as do protein disulfide isomerase (PDI) or prolyl-peptidyl cis/trans isomerase (see Freedman, 1989). Instead, chaperones have been hypothesized to aid protein folding by binding nascent polypeptide chains and thereby preventing improper but thermodynamically stable structures from forming until translation can be completed (Ellis and Hemmingsen, 1989; Rothman, 1989; Gething and Sambrook, 1990). This function requires that chaperones recognize and bind specific structural motifs exposed in nascent polypeptides but not in mature polypeptides. In vitro binding studies using GRP78-BiP and hsc70 have indicated that these molecules bind peptides with some degree of sequence-specificity and that release is dependent on ATP hydrolysis (Flynn *et al.*, 1989). Our studies presented here confirm those results and are consistent with the proposed function of chaperone proteins. By creating a series of deletion mutants using the SV5 HN polypeptide, we showed that in vivo GRP78-BiP interactions show selectivity and that GRP78-BiP interacts with at least two different sites on HN. However, the nature of the deletions makes it possible that within these large segments of HN more than one site is available to interact with GRP78-BiP, but these remain to be determined. The existence of multiple sites is consistent with the idea that chaperones bind nascent chains to maintain an open conformation favorable for folding. However, we have no knowledge as to whether the sites of interaction of GRP78-BiP with HN should be considered as linear domains of amino acids or if they have specific conformations and form recognition patches. Previous studies focusing on Ig heavy chains (HC) have suggested that at least two domains, C_H1 and V_H, can be used to bind GRP78-BiP. Naturally occurring deletions of these domains reduce the association with GRP78-BiP and allow the secretion of HC in the absence of light-chain synthesis (Hendershot *et al.*, 1987; Pollock *et al.*, 1987). However, these observations should not be necessarily interpreted to suggest that GRP78-BiP is absent from the process of folding of mutant HC because either deletion mutant may continue to contain the other available binding sites.

Newly synthesized molecules in the ER must properly fold and assemble before transport to the Golgi apparatus (Gething *et al.*, 1986; Copeland *et al.*, 1986). Although there is mounting evidence that molecular chaperones such as GRP78-BiP are involved in the folding of polypeptides, it must be stressed that additional factors may be essential in promoting folding of proteins in the secretory pathway. Unlike the cytosol, the ER lumen is an oxidizing environment where intramolecular and intermolecular disulfide bonds may form between cysteine residues. In the native molecule, appropriate disulfide bonds can stabilize conformational structures, whereas inappropriate disulfide bonds can lead to irreversible malfolded "dead-end" products (Machamer and Rose, 1988b; Hurtley *et al.*, 1989). Correct disulfide bond formation requires the lumenal enzyme PDI, which catalyzes thiol:disulfide interchange reactions (Bulleid and Freedman, 1988; reviewed in Freedman, 1989). Lumenal proteins are also subject to modification by N-linked glycosylation that may also affect folding because underglycosylated or improperly glycosylated proteins are often misfolded (Gibson *et al.*, 1979; Gallagher *et al.*, 1988; Machamer and Rose, 1988a), e.g., underglycosylated HN fails to properly fold despite the presence of functional GRP78-BiP (Ng *et al.*, 1990).

GRP78-BiP transcription is activated by a large variety of perturbants, most of which affect activities in the ER where GRP78-BiP is localized (see INTRODUCTION). Most notable is the effect caused by the expression of

malfolded proteins in the ER where the induction is independent of potential pleiotropic effects caused by chemicals (Kozutsumi *et al.*, 1988). The nature of the signaling pathway from the ER to the nucleus to activate transcription of GRP78-BiP is unknown; however, the stimulus that activates this pathway is becoming clear. It has been found that the increased synthesis of secretory proteins that causes induction of GRP78-BiP is most likely due to the increase in substrate in the ER (Dorner *et al.*, 1989). In addition, it has been shown in cells infected with paramyxoviruses that there is a twofold induction in GRP78-BiP synthesis that is due to the expression of the HN protein (Watowich *et al.*, 1991). However, the expression of another glycoprotein influenza HA, using the same expression system, did not cause an induction in GRP78-BiP synthesis (Kozutsumi *et al.*, 1988). It has been suggested that as HN folds relatively slowly ($t_{1/2}$, 25–30 min) (Ng *et al.*, 1989) as compared with HA ($t_{1/2}$, 7–10 min) (Gething *et al.*, 1986) and that as GRP78-BiP has a prolonged transient association with HN during the process of folding (Ng *et al.*, 1989), this reduces the unbound pool of GRP78-BiP in the ER, thus requiring an induction of GRP78-BiP synthesis to maintain the pool of GRP78-BiP molecules (Watowich *et al.*, 1991). The studies presented here extend the suggestion (Gething and Sambrook, 1990; Watowich *et al.*, 1991) that a prolonged association with substrates is the basis of GRP78-BiP induction. HNT_2 was found to be associated stably with GRP78-BiP and resulted in a greater induction than HN, which associates with GRP78-BiP transiently, whereas mutant proteins that did not bind GRP78-BiP, but are considered nonnative structures and are retained in a pre-Golgi apparatus compartment, did not cause an induction in GRP78-BiP synthesis. These data point to a mechanism where the levels of free GRP78-BiP are monitored and the expression is adjusted depending on the needs of the cell. Further evidence supporting such a feedback mechanism comes from studies with a yeast strain in which the HDEL C-terminal ER retention signal on GRP78-BiP was deleted. Although GRP78-BiP was secreted, the cells were able to grow normally due to a massive increase in the synthesis of GRP78-BiP, apparently to compensate the loss due to secretion. When compared with wild-type cells, the intracellular levels were found to be identical (Hardwick *et al.*, 1990).

To monitor the levels of GRP78-BiP/protein, bound and unbound forms of GRP78-BiP have to be distinguished. A possible indicator as to how this is accomplished comes from studies analyzing post-translational modifications of GRP78-BiP. Unbound GRP78-BiP was found to be phosphorylated and ADP-ribosylated, whereas GRP78-BiP associated with Ig heavy chains was unmodified (Hendershot *et al.*, 1988), and thus modified GRP78-BiP may be used to repress components of the signaling pathway. The molecular basis for the transduction of a signal from the ER to the nucleus is unknown. Whether it is similar to signaling pathways originating from the plasma membrane remains to be determined.

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