BiP, a Major Chaperone Protein of the Endoplasmic Reticulum Lumen, Plays a Direct and Important Role in the Storage of the Rapidly Exchanging Pool of Ca\(^{2+}\)^*  

(Received for publication, March 7, 1997, and in revised form, September 4, 1997)

Jean-Philippe Liévremont†, Rosario Rizzuto§, Linda Hendershot¶, and Jacopo Meldolesi†‡  

From the †Department of Biomedical Sciences and CNR Center for the Study of Mitochondrial Physiology, University of Padova, 35121 Padova, Italy, ‡Department of Tumor Cell Biology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38101, and †Department of Pharmacology and B. Ceccarelli Center of Neurobiology, University of Milano, CNR Center of Molecular and Cellular Pharmacology, and DIBIT, Scientific Institute San Raffaele, 20132 Milano, Italy

The activity of BiP, the major chaperone of the endoplasmic reticulum (ER) lumen, is known to be Ca\(^{2+}\)-regulated; however, the participation of this protein in the ER storage of the cation has not yet been investigated. Here such a role is demonstrated in human epithelial (HeLa) cells transiently transfected with the hamster BiP cDNA and incubated in Ca\(^{2+}\)-free medium, as revealed by two different techniques. In the first, co-transfected aequorin was employed as a probe for assaying either the cytosolic of the mitochondrial free Ca\(^{2+}\) concentration. By this approach higher Ca\(^{2+}\) release responses were revealed in BiP-transfected cells by experiments in which extensive store depletion was induced either by repetitive stimulation with inositol 1,4,5-trisphosphate-generating agonists or by treatment with the Ca\(^{2+}\) ionophore, A23187. In the second technique the cells were loaded at the equilibrium with \(\text{Ca}^{45}\), and the release of the tracer observed upon treatment with thapsigargin, a blocker of the ER Ca\(^{2+}\) ATPases, was larger in BiP-transfected than in control cells. The latter results were obtained also when BiP was overexpressed not via transfection but as a response to ER stress by tunicamycin. These results are sustained by increases of the ER Ca\(^{2+}\) storage capacity rather than by artifacts or indirect readjustments induced in the cells by the overexpression of the chaperone since (a) the exogenous and endogenous BiP were both confined to the ER, (b) the expression levels of other proteins active in the ER Ca\(^{2+}\) storage were not changed, and (c) effects similar to those of wild type BiP were obtained with a deletion mutant devoid of chaperone activity. The specificity of the results was confirmed by parallel \(\text{Ca}^{45}\) experiments carried out in HeLa cells transfected with two other Ca\(^{2+}\)-binding proteins, calreticulin and CaBP\(_2\) (ERp72), only the first of which induced increases of Ca\(^{2+}\) capacity. We conclude that BiP has a dual function, in addition to its chaperone role it is a bona fide ER luminal Ca\(^{2+}\) storage protein contributing, under resting cell conditions, to around 25% of the store, with a stoichiometry of 1–2 moles of calcium/mole of BiP.

Studies carried out during the last decade have identified BiP as a ubiquitous luminal resident protein of the endoplasmic reticulum (ER)\(^{1,3}\), playing a key role in the assistance of newly synthetized proteins for folding and acquisition of their correct tertiary and quaternary structure. Such a function, defined as a chaperone, implies the direct binding of BiP to the growing chains, with a stimulation of its ATPase activity (6–9). When this task is not accomplished, misfolded proteins remain complexed to BiP (and to other chaperones) (7, 10) to be ultimately disposed of by nonlysosomal proteolytic process(es) (quality control) (7, 9–11). These fundamental activities are carried out by BiP working in the peculiar environment of the ER lumen (12) characterized, among other properties, by a high (mM) concentration of free Ca\(^{2+}\) (\([\text{Ca}^{2+}]_{\text{ER}}\)) (13, 14). Such a property of the environment appears to be of importance for BiP function. Indeed, previous studies carried out with both recombinant BiP protein and BiP purified from cells have shown that the chaperone ATPase activity is altered by Ca\(^{2+}\), and BiP association with proteins in vitro is stabilized by Ca\(^{2+}\) (8, 15, 16). Moreover, major changes of Ca\(^{2+}\) homeostasis, such as those induced by prolonged applications of Ca\(^{2+}\) ionophores, have been shown to induce major disturbances in the cells, including inappropriate release and secretion of BiP-associated proteins (17), alterations in the folding of ER proteins leading to the transcriptional up-regulation (3, 18), and altered distribution of BiP and other chaperones (19). So far, however, the possibility that BiP, in addition to its chaperone function, could also participate in the intraluminal storage of Ca\(^{2+}\), thus contributing to the accumulation of the rapidly exchanging pool of the cation, has never been investigated.

In the present study we report that overexpression of exogenous hamster BiP in HeLa cells induces appreciable increases of the ER Ca\(^{2+}\) storage capacity as consistently revealed by two experimental approaches. In one, cells were cotransfected with the cDNA of the photoprotein aequorin that had been molecularly modified to assay \([\text{Ca}^{2+}]_{\text{ER}}\) within two distinct intracellular compartments, either the cytosol or the mitochondria (13, 20). In the other, the cells were loaded at the equilibrium with \(\text{Ca}^{45}\) and release was induced by exposure to thapsigargin (Tg), a blocker of the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) pump (SERCA). The latter treatment is known to induce the specific discharge of the ER compartment (13, 21). The Ca\(^{2+}\) storage effects observed with BiP overexpression were not due to its chaperone activity toward other proteins, since a BiP construct

* This work was carried out in the framework of the European Community TMR network program (fellowship to J. P. L.) and was also supported by grants from Italian Telethon (652), the International Human Frontier Science Program (RG-520/95), and United States National Institutes of Health Grant GM 54068. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ”advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: DIBIT, H. San Raffaele, via Olgettina, 58, 20132 Milano, Italy. Tel: 39 2 2643 2770; Fax: 39 2 2643 4813; E-mail: meldolj@dibit.hsr.it.

‡ The abbreviations used are: ER, endoplasmic reticulum; IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_3\)R, IP\(_3\) receptor; Tg, thapsigargin; SERCA, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) pump; PDI, protein disulfide isomerase; CNX, calnexin; CRT, calreticulin; PBS, phosphate-buffered saline; KKH, Krebs-Ringer-Hepes.
that lacks the protein binding domain produced similar results. Nor were these effects due to compensatory readjustments in the levels of other ER Ca\(^{2+}\)-binding proteins, because the latter remained unchanged in the transfected cells. Moreover, parallel studies carried out in HeLa cells exposed to a stressful treatment with the glycosylation blocker drug, tunicamycin (22), also led to an increase of the ER Ca\(^{2+}\) storage capacity, part of which is dependent on the stress-induced overexpression of BiP. We conclude that, in addition to its classical role of chaperone, BiP participates in a second function, the storage of Ca\(^{2+}\) within the ER lumen, and that the fluctuations of the protein induce adjustments of the cellular Ca\(^{2+}\) homeostasis.

**EXPERIMENTAL PROCEDURES**

**Materials**—HeLa cells line were obtained from ATCC, Rockville, MD; Tg from LC Service, Woburn, MA. The antibodies employed have been described elsewhere: anti-hamster BiP; a rabbit polyclonal specific for the rodent protein (23); and a rat monoclonal antibody which recognizes both the rodent and human BiP (1); anti-CRT and anti-CaBP\(_2(ErP72)\), two rabbit polyclonals (24); anti-protein disulfide isomerase (PDI), a rabbit polyclonal (25); anti-inositol 1,4,5-trisphosphate (IP\(_3\)) receptor, a rabbit polyclonal (26); and anti-calnexin (CNX), a rabbit polyclonal (27). 1.25-Labeled protein A, ECL kit, and \(^{44}\)Ca were purchased from Amersham Int., United Kingdom; antibodies from Eukaryotic Expression Vectors, Inc., Basel, Switzerland; ATP, histamine, A23187, tunicamycin, and the remaining chemicals from Sigma-Aldrich, Milan, Italy.

**Eukaryotic Expression Vectors**—The eukaryotic expression vectors employed for the transfection experiments have been described elsewhere. The pMT vectors containing the cDNA of the wild-type hamster BiP (wt-BiP/pMT) and of the 44K amino-terminal ATPase domain (lacking the protein binding domain but retaining the carbonyl-terminal 11 amino acids required for recognition by the anti-BiP antiseraum and for ER retention: 44K\mbox{BiP/pMT}) were described elsewhere (23); both the pcDNAI vectors containing either the cDNA of aquorin targeted to either the cytosol (cytAEQ/pcDNAI) or to mitochondria (mtAEQ/pcDNAI) as well as the cDNA of CRT (tCR/pcDNAI) were described in Bastianutto et al. (13) and Briani et al. (28); the pCMV vector including the cDNA of rat CaBP\(_2(ErP72)\) (CaBP\(_2(ErP72)/pCMV2\) was described in Van et al. (29).

**Cell Culture and Transfection**—HeLa cells were grown at 37 °C in 75-cm\(^2\) Falcon flasks, bathed in Dulbecco modified Eagle’s medium supplemented with 10% fetal calf serum and 100 units/ml penicillin and streptomycin, under a 5% CO\(_2\) atmosphere. Transient expression of plasmid DNA (cytAEQ/pcDNAI or mtAEQ/pcDNAI) as well as the cDNA of CRT (tCR/pcDNAI) were described in Bastianutto et al. (13) and Briani et al. (28); the pCMV vector including the cDNA of rat CaBP\(_2(ErP72)\) (CaBP\(_2(ErP72)/pCMV2\) was described in Van et al. (29).

**Cell Stress Experiments**—In the stress experiments monolayers of HeLa cells were grown as described above, except that during the last 48 h their incubation medium was supplemented with \(^{44}\)Ca (1.5 μCi/ml). At the end of this period the labeled cells were rapidly washed in Krebs-Ringer-Hepes (KRH) medium containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 6 mM glucose, 25 mM Hepes-NaOH, pH 7.4). The cells were then resuspended at 37 °C in 3 mM EGTA-containing Ca\(^{2+}\)-free KRH medium, and Tg (0.1 μM in Me\(_2\)SO) was added quickly. Immediately before and 4 min after the addition of Tg, aliquots of 1 × 10\(^6\) cells were removed and centrifuged. The \(^{44}\)Ca recovered from the medium and the pellets were assayed in a Beckman \(\beta\)-counter (for further details, see Fasold et al. (35)).

## RESULTS

**Characterization of BiP-transfected HeLa Cells**—The HeLa cell system employed for the reported experiments was systematically characterized by biochemical and immunocytochemical studies. Fig. 1 shows confocal images of cells, transfected with either the wild type or the 44K amino-terminal portion of hamster BiP, that have been decorated with a purified polyclonal antibody raised against the 11-amino acid COOH-termini-
The decoration of Western blots from transfected cells, carried out with the same antibody employed for immunocytochemistry, is shown in Fig. 2A. Notice that with the polyclonal antibody control cells were negative while the bands corresponding to the wild type and the 44KΔBiP-transfected proteins were prominent. Fig. 2B shows in contrast the bands of control and wild type BiP-transfected cells revealed by the rat monoclonal antibody, which recognizes both rodent and human BiP in control (1) and wild type BiP-transfected (2) cells. To the right the calibration scale of purified recombinant hamster protein (rBiP) shows the bands obtained with the same antibody and (from left to right) with 50, 100, 250, 500, and 1000 ng of the protein. Panel C shows the blots of control (left) and wild type BiP-transfected (right) cells labeled by antibodies specific for the following ER proteins: PDI, CNX, CRT, and IP3R. Panel D shows the blots of control (left) and CaBP2 (ERp72)-transfected (right) cells revealed by the polyclonal antibody specific for rat CaBP2 (ERp72). Notice the prominent single band detected in the transfected cells (right lane, apparent molecular mass, 80 kDa), while in control cells no signal is appreciable (left lane).

Fig. 2. Western blot analysis of control and BiP-transfected cells with antibodies specific for either BiP or different ER proteins. Equal amounts of proteins from HeLa cells, controls, and transfected with either the wild type hamster BiP or its deletion mutant 44KΔBiP, were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. Panel A shows the bands labeled with the polyclonal antibody raised against the COOH-terminal tail of hamster BiP, specific for the rodent protein. Notice the negativity of control cells (1) and the bands appearing with the wild type (2) and 44KΔBiP (3) transfected preparations (apparent molecular mass of 78 and 44 kDa, respectively). Panel B shows the blots revealed by the rat monoclonal antibody, which recognizes both human and rodent BiP in control (1) and wild type BiP-transfected (2) cells. To the right the calibration scale of purified recombinant hamster protein (rBiP) shows the bands obtained with the same antibody and (from left to right) with 50, 100, 250, 500, and 1000 ng of the protein. Panel C shows the blots of control (left) and wild type BiP-transfected (right) cells labeled by antibodies specific for the following ER proteins: PDI, CNX, CRT, and IP3R. Panel D shows the blots of control (left) and CaBP2 (ERp72)-transfected (right) cells revealed by the polyclonal antibody specific for rat CaBP2 (ERp72). Notice the prominent single band detected in the transfected cells (right lane, apparent molecular mass, 80 kDa), while in control cells no signal is appreciable (left lane).

The stray stretches of the hamster protein. This antibody is specific for rodent BiP and does not recognize the human protein endogenous to HeLa cells (23) (see also Fig. 2A). From the immunocytochemical results, the percentage of transfected HeLa cells was established to vary in different experiments from 35 to 50%. Among positive cells, those heavily expressing the transfected protein were relatively rare, while most exhibited levels that remained well below saturation under the employed experimental conditions. Moreover, the distribution of both wild type and 44KΔBiP proteins revealed the classical, ER-type reticular pattern (Fig. 1, B and C), similar to that given by other luminal proteins such as CRT (not shown; see Bastianutto et al. (13)). Further characterization of the BiP-overexpressing population was carried out at the electron microscope level. In these experiments thin sections of at least 20 cells group were analyzed, with special interest for the extension and distribution of the ER and for the size and shape of its cisternae. Such numbers guarantee the study of both transfected and non-transfected cells. An ultrastructural analysis was important because in a previous study the cells transfected with a BiP mutant (but not with the wild type or the 44KΔBiP mutant employed here) were reported to exhibit fragmented cisternae (23), a typical sign of cell damage. In our samples, however, significant alterations of the ER were never observed (not shown). We conclude therefore that transfection of both wild type and 44KΔ mutant BiP did not induce any unspecific cell damage appreciable by conventional electron microscopy.

The preparation for rodent BiP and does not recognize the human protein endogenous to HeLa cells (23) (see also Fig. 2A). From the immunocytochemical results, the percentage of transfected HeLa cells was established to vary in different experiments from 35 to 50%. Among positive cells, those heavily expressing the transfected protein were relatively rare, while most exhibited levels that remained well below saturation under the employed experimental conditions. Moreover, the distribution of both wild type and 44KΔBiP proteins revealed the classical, ER-type reticular pattern (Fig. 1, B and C), similar to that given by other luminal proteins such as CRT (not shown; see Bastianutto et al. (13)). Further characterization of the BiP-overexpressing population was carried out at the electron microscope level. In these experiments thin sections of at least 20 cells group were analyzed, with special interest for the extension and distribution of the ER and for the size and shape of its cisternae. Such numbers guarantee the study of both transfected and non-transfected cells. An ultrastructural analysis was important because in a previous study the cells transfected with a BiP mutant (but not with the wild type or the 44KΔBiP mutant employed here) were reported to exhibit fragmented cisternae (23), a typical sign of cell damage. In our samples, however, significant alterations of the ER were never observed (not shown). We conclude therefore that transfection of both wild type and 44KΔ mutant BiP did not induce any unspecific cell damage appreciable by conventional electron microscopy.

The decoration of Western blots from transfected cells, carried out with the same antibody employed for immunocytochemistry, is shown in Fig. 2A. Notice that with the polyclonal antibody control cells were negative while the bands corresponding to the wild type and the 44KΔBiP-transfected proteins were prominent. Fig. 2B shows in contrast the bands of control and wild type BiP-transfected cells revealed by the rat monoclonal antibody, which recognizes both the rodent (exogenous) and human (endogenous) protein, as well as a scale of bands obtained with increasing amounts of purified recombinant hamster BiP protein. Based on the calibration curves drawn from these (and other) results, the total level of BiP expression in the transfected cell population was estimated systematically in all experiments. On the average, such a level was found to be ~200% of that in controls. Fig. 2C shows blots of control and BiP-transfected cells decorated with antibodies raised against other ER proteins, the chaperones PDI, CNX and CRT, the latter being also the major Ca2+ -binding protein of the system (34, 35); as well as the IP3 receptor. As can be seen (Fig. 2C), none of these components appeared appreciably different in the BiP-transfected preparations with respect to controls, suggesting that no significant changes in the expression of other ER proteins that contribute to Ca2+ homeostasis had taken place.
Role of BiP in ER Ca\textsuperscript{2+} Storage

FIG. 3. Ca\textsuperscript{2+} release to the HeLa cell cytosol elicited by A23187 and revealed by cytosolic aequorin. Three superimposed traces (panel A) illustrate the typical [Ca\textsuperscript{2+}]\textsubscript{c}, increases induced by the Ca\textsuperscript{2+} ionophore A23187 (10 μM) administered to HeLa cells transfected with cytosolic aequorin, either alone (control) or together with either wild type BiP or 44KΔBiP, incubated in Ca\textsuperscript{2+}-free KRH medium supplemented with 100 μM EGTA. Calibration of the aequorin signal into [Ca\textsuperscript{2+}]\textsubscript{c} values was carried out as described in Brini et al. (28). The analyses of the A23187-induced [Ca\textsuperscript{2+}]\textsubscript{c} responses, such as those shown in the traces, are presented in the bar panels (B) in terms of both [Ca\textsuperscript{2+}]\textsubscript{c} peak (left) and total response areas (right). Values shown are averages ± S.D. of the six separate experiments.

\[[Ca^{2+}]_c\] and \[[Ca^{2+}]_mt\] Assays with Aequorin—The effects of increased levels of BiP expression on Ca\textsuperscript{2+} release from the ER compartment, as revealed by the cotransfected photoprotein, aequorin, trapped within the cytosol, are shown in Figs. 3 and 4. Control and BiP-overexpressing cells, bathed in the Ca\textsuperscript{2+}-free medium, were exposed to the Ca\textsuperscript{2+} ionophore, A23187 (Fig. 3A). The signal generated by this treatment originates, however, not only from the ER, but also from any other stores with nonacidic lumena. Despite this limitation of the approach, in six out of six experiments the release signals from the cell populations with wild type BiP (and, to a slightly lower level, also with the 44KΔBiP) were greater than those from controls, both in terms of maximal [Ca\textsuperscript{2+}]\textsubscript{c} peak and of total [Ca\textsuperscript{2+}]\textsubscript{c} response area (see the representative trace results in Fig. 3A and the average ± S.D. of the six experiments in Fig. 3B).

In a subsequent series of experiments, the study of the cells cotransfected with BiP and aequorin was focused on the receptor, IP\textsubscript{3}-mediated responses of cytosolic free Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}]\textsubscript{c}, triggered by release of the cation from the ER. Previous studies of HeLa cells transfected with another luminal Ca\textsuperscript{2+} binding protein, CRT (13), had shown that the signaling differences sustained by differences in the capacity of the ER store are hard to reveal in response to a single shot given to cells, and become much more clear when the cells, incubated in Ca\textsuperscript{2+}-free medium to prevent the contribution of Ca\textsuperscript{2+} influx, are exposed sequentially to two short (30 s) treatments with agonists specific for different receptors (to prevent desensitization), first ATP and then histamine (Fig. 4) or vice versa (not shown).

Similarly to what is observed with CRT (13), cells transfected with either wild type BiP or 44KΔBiP were found in five experiments to show an initial [Ca\textsuperscript{2+}]\textsubscript{c} increase only moderately greater and more persistent than that of controls. In contrast, in the second response the difference became remarkable in all the experiments because the [Ca\textsuperscript{2+}]\textsubscript{c} rise was greatly reduced in the controls and still considerable in the transfected cells (on the average +150 ± 12% with wild type BiP). These last results document the persistence of a considerable Ca\textsuperscript{2+} pool within the ER stores of transfected cells at a time when in controls these stores were largely depleted.

The question which was then raised was whether the increased [Ca\textsuperscript{2+}]\textsubscript{c} responses such as those observed in the BiP-transfected cells were restricted to the cytosol or could have a more vast impact on cellular Ca\textsuperscript{2+} homeostasis. To investigate the problem, experiments were carried out by applying the same two response protocol used in Fig. 4, but this time to cells that had been cotransfected with an aequorin-containing construct addressed not to the cytosol but to mitochondria, the organelles known to depend largely from ER release for their Ca\textsuperscript{2+} uptake (20). The results obtained by this approach, concerning cells transfected with either BiP or CRT, are shown in Fig. 5. In terms of size, the rises of mitochondrial free Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}]\textsubscript{mt}, in the BiP-transfected cells in response to the first stimulation were hardly different from those in controls, whereas those of the CRT-transfected cells were moderately increased. Persistence of these responses was slightly prolonged in both cases. Clear differences emerged, however, with the second stimulation. In control cells the [Ca\textsuperscript{2+}]\textsubscript{mt} responses were in fact decreased with respect to the first, although less markedly than those of [Ca\textsuperscript{2+}]\textsubscript{c} (cf. Figs. 4 and 5). In contrast, these second responses were largely maintained in the cells transfected with either luminal Ca\textsuperscript{2+} binding protein (Fig. 5).

We conclude that the increased capacity for Ca\textsuperscript{2+} of the ER can extend its impact on signaling to mitochondria where the cation is known to regulate various dehydrogenases and through them the entire Krebs cycle (36).

\textsuperscript{45}Ca Release—Further information about the functioning of the ER Ca\textsuperscript{2+} stores in the BiP-transfected cells was obtained by assaying \textsuperscript{45}Ca release after cell equilibrium loading with the isotope and exposure not to a ionophore but to the specific SERCA blocker, Tg. In this case the analyzed \textsuperscript{45}Ca release is
and CaBP2(ERp72)-transfected HeLa cells, revealed by the number of the chaperone PDI family (29, 37, 38). Blots of control experiments and presentation are the same as in Fig. 4 except that (a) the analyzed cells were transfected with either wild type BiP or CRT, (b) the cotransfected aequorin was addressed to mitochondria, and (c) the interval between the stimulations was 10 (instead of 1.5) min to give enough time for the mixing of aequorin within mitochondria. Results shown are representative of three experiments. HIS, histamine.

FIG. 5. Ca\(^{2+}\) rises in mitochondria of control and transfected HeLa coupled to IP\(_3\) generation. Experiments and presentation are the same as in Fig. 4 except that (a) the analyzed cells were transfected with either wild type BiP or CRT, (b) the cotransfected aequorin was addressed to mitochondria, and (c) the interval between the stimulations was 10 (instead of 1.5) min to give enough time for the mixing of aequorin within mitochondria. Results shown are representative of three experiments. HIS, histamine.

FIG. 6. \(^{45}\)Ca release from HeLa cells first transfected with BiP, CRT, or CaBP\(_2\)(ERp72) and then loaded at the equilibrium, to be then exposed to Tg. The graph represents the \(^{45}\)Ca release to the medium from suspensions of HeLa cells, controls, or transfected with either wild type BiP, CRT, or CaBP\(_2\), induced by a 4-min treatment with Tg (0.1 \(\mu\)M). Cells were cultured for 2 d in presence of the \(^{45}\)Ca isotope and then incubated in Ca\(^{2+}\)-free KRH medium supplemented with 3 mM EGTA. Expression of the three proteins with respect to controls was assessed in parallel by quantitative Western blotting. 100% release corresponds to 620 cpm/mg of cell protein. Results shown are averages \(\pm\) S.D. of two highly consistent experiments performed in triplicate.

FIG. 7. \(^{45}\)Ca release from nontransfected HeLa cells loaded at the equilibrium and stressed with tunicamycin (TM), when exposed to Tg. Experimental conditions and presentation as in Fig. 6 except that 1) all cells analyzed were nontransfected, and 2) loading with \(^{45}\)Ca was for 72 h, during the last 24 h of which half of the cells were exposed to tunicamycin (7 \(\mu\)g/ml in Me\(_2\)SO) while the others received the solvent only. The Western blots of BiP and CRT in stressed and control (C) cell populations are shown to the left. 100% release corresponds to 550 cpm/mg of protein. Results shown are averages \(\pm\) S.D. of two highly consistent experiments performed in triplicate.

Role of BiP in ER Ca\(^{2+}\) Storage

Expected to originate from the ER store of all cells, and thus from both transfected and untransfected cells. Despite this limitation in the experimental approach, the Tg-induced release observed from the BiP-transfected population was distinctly greater as compared with controls as shown in Fig. 6. The same figure shows that even bigger increases could be obtained from HeLa cells transfected with CRT (total level of expression, 250%; see also Bastianutto et al. (13)). Parallel experiments were carried out with another Ca\(^{2+}\)-binding protein, CaBP\(_2\), the rat homologue of the murine ERp72, a member of the chaperone PDI family (29, 37, 38). Blots of control and CaBP\(_2\)(ERp72)-transfected HeLa cells, revealed by the antibody raised against the rat protein, showed a weak signal in control cells. In contrast, a prominent band was detected in the CaBP\(_2\)(ERp72)-transfected cells (Fig. 2D), demonstrating that expression of the protein was considerably increased. Despite this result, the capacity of the Tg-sensitive store, as revealed by \(^{45}\)Ca experiments, was not increased, but rather slightly lower in CaBP\(_2\)-transfected cells with respect to controls (Fig. 6). Thus, appreciable increases of ER capacity for Ca\(^{2+}\) do not necessarily follow the overexpression of any ER luminal Ca\(^{2+}\)-binding protein.

Increased expression of BiP can also occur in cells independent of cDNA transfection, being a well known aspect of the cell stress response. In view of the observed consequences of BiP overexpression on Ca\(^{2+}\) homeostasis we were lent to investigate whether ER stress was also able to appreciably modify the Ca\(^{2+}\) capacity of the ER and if so, whether BiP was involved. To carry out these experiments the cells were exposed to the N-glycosylation blocker, tunicamycin (22), applied after equilibration of Ca\(^{2+}\) pools with \(^{45}\)Ca. Compared with other stressful treatments, such as those with A23187 and Tg, treatment with tunicamycin was found to be particularly appropriate for our purposes since by itself the drug does not appear to have any direct effect on Ca\(^{2+}\) homeostasis, whereas it does induce considerable increases of the ER chaperones, in particular of those that also serve as luminal Ca\(^{2+}\) binding proteins, such as BiP (22) and CRT (39). Increases of these proteins, appreciable as early as 6 h, were found to reach plateaus after 24 h, with average values in our experiments of 58% for CRT and 100% for BiP (Fig. 7A). Not surprisingly, these increases in expression were paralleled by marked increases (68%) of the \(^{45}\)Ca release responses induced by Tg (Fig. 7B).

Calculations, carried out as described in Bastianutto et al. (13), based, on the one hand, on the levels of expression of the two proteins as established by Imagequant analysis of the Western blots developed from fractions of the same cell lysis used for the \(^{45}\)Ca release experiments, and on the other hand on the resting Ca\(^{2+}\) storage data in the ER of both control and transfected cells (Fig. 6) (13), revealed the contribution of CRT to account for ~29% and that of BiP for ~25% of the increased ER Ca\(^{2+}\) capacity induced by Tg treatment.
DISCUSSION

The present results, obtained by two independent techniques, consistently reveal increased capacity of the rapidly exchanging ER Ca\(^{2+}\) store in the well characterized system of HeLa cells transiently transfected with either wild type BiP or its deletion mutant, 44K\(\Delta\)BiP. The two techniques employed should be considered complementary of each other. Cotransfection of the cytosol-targeted aequorin (28) is advantageous not only because it provides the time course of the induced Ca\(^{2+}\) release responses but also because the origin of the Ca\(^{2+}\)-triggered signal is restricted to cells expressing the photoprotein, which largely coincide with those overexpressing the cotransfected protein, i.e. the wild type or the 44K\(\Delta\)BiP. This property is important because in our transfected preparations only 35–50% of the cells expressed exogenous BiP on top of their endogenous complement of the protein. However, the aequorin signals depend not only on the Ca\(^{2+}\) capacity, but also on other properties of the ER store (pumps, channels) as well as on homeostatic equilibria established within the cell. Therefore, they cannot be easily converted into quantitative data. 

\(^{45}\)Ca release, on the other hand, occurs from all the cells of the preparation (transfected and non transfected) which were first loaded at the equilibrium with the tracer. The advantage here is that, by using Tg, a blocker specific for the SERCAs, the results obtained are due to Ca\(^{2+}\) release only from the ER (13).

The significance of our findings deserves attention. As in all other overexpression experiments, results could in fact be due not (or not only) to the increased amounts of the investigated protein but (also) to indirect consequences induced by protein transfection. In the case of BiP, this concern appears particularly cogent in view of the chaperone function of this protein, with positive action on structure, and thus also on function, of other proteins, some of which contribute to ER Ca\(^{2+}\) homeostasis. However, the control results we have obtained do not seem to support these possibilities. The level of other proteins of the ER store, including CRT, which is known to be the major Ca\(^{2+}\) binder (34, 35), was in fact not significantly different in BiP-transfected and control cells. Changes of the ER store capacity due not to BiP but to BiP-induced changes of expression of other components appear therefore unlikely. Likewise, the consistence of the reported results obtained not only with BiP but also with its 44K\(\Delta\)BiP mutant, which is devoid of the peptide binding domain and thus of any chaperone function (23), excludes the involvement of a chaperone-induced improved function of the other Ca\(^{2+}\)-binding proteins. It appears therefore that the effects observed are most likely due to direct, specific Ca\(^{2+}\) binding of the transfected protein. This conclusion is reinforced by the additional \(^{45}\)Ca data obtained using cells transfected not with BiP but with either CRT or CaBP\(_2\) (ERp72). The results obtained on the one hand confirm that the contribution of CRT to the HeLa cell rapidly exchanging Ca\(^{2+}\) store amounts to \(\sim 25\%\) (13) and on the other hand fail to show any detectable effect of CaBP\(_2\). In the cells overexpressing the latter protein, in fact, the apparent capacity of the ER in terms of Ca\(^{2+}\) storage was not increased but, if anything, slightly reduced.

A role of BiP as one of the Ca\(^{2+}\) storage proteins of the ER lumen was proposed 10 years ago by Macer and Koch (40) who first observed a polyacrylamide gel electrophoresis band with an apparent molecular mass of \(\sim 78\) kDa positively labeled by \(^{45}\)Ca overlay. Subsequent studies by Van et al. (41), carried out by a more ample approach, revealed, however, that the \(^{45}\)Ca-positive band of that size was not BiP but CaBP\(_2\)(ERp72). In the meantime, evidence was accumulated demonstrating that for its chaperone function, in particular for the binding of proteins and ATPase activity (8, 15, 16), BiP needs Ca\(^{2+}\) to be present in the medium. So far, however, the possibility that BiP contributes to the storage of the cation within the ER lumen, together with other proteins such as CRT, had never been envisaged. By the use of the aequorin approach in HeLa cells we demonstrate here that the contribution of the chaperone to the capacity of the typical rapidly exchanging Ca\(^{2+}\) pool is not insignificant. In fact, when investigated according to a two-stimulation protocol (13), BiP overexpression was found to induce detectable increases of the Ca\(^{2+}\) release triggered by IP\(_3\) generation following receptor activation. This type of difference is not easy to evidence because it is extensively buffered by cytotoxic and organelle mechanisms (13, 42). Moreover, extension of the approach to mitochondria, carried out by studying cells expressing an aequorin construct addressed to those organelles (20), revealed that the BiP contribution to the ER-segregated Ca\(^{2+}\) pool has a wide impact within the cell. In fact, similar to the cells overexpressing CRT, those transfected with BiP were able to show considerable \([\text{Ca}^{2+}]_{\text{ER}}\) transient also in response to the second stimulation, when the responses of control cells were clearly reduced. In view of the importance of Ca\(^{2+}\) in mitochondrial physiology (36) these observations further document the importance of the BiP storage function in cell physiology.

Interesting conclusions can be drawn also from the \(^{45}\)Ca results. The \(\sim 25\%\) increases of the Tg-induced release obtained from the transfected preparations, in which the overall BiP expression was twice as large as in the controls, suggest that the contribution of the endogenous chaperone corresponds to \(\sim 25\%\) as well. To reconstruct the whole ER segregated Ca\(^{2+}\) pool the BiP value should be added to that previously estimated for CRT, \(\sim 50\%\), with the rest probably contributed by other components, not only proteins (lumenal and also of the membrane, such as CNX) but also nucleotides (42). Taking into account that in HeLa cells the concentration of BiP is considerable (\(\sim 1\%\) of the protein, \(\sim 5\)-fold higher than CRT), the above results suggest that the stoichiometry of ER lumenal Ca\(^{2+}\) binding, under resting conditions, is relatively low, i.e. between 1 and 2 moles of calcium/mole of protein. Since in the BiP sequence typical Ca\(^{2+}\) binding domains are not evident, it appears likely that the observed binding is due to the abundance of acidic amino acids, many of which arranged in doublets or triplets (43, 44). Because of its role in Ca\(^{2+}\) accumulation and release, and by analogy with other storage proteins such as CRT and calsequestrin (42), this binding is expected to be of low affinity, although so far conclusive results with the purified protein have not been reported.

A final comment concerns the results obtained with cells that, instead of being transfected, were exposed to tunicamycin to induce stress (27). Under these conditions, overexpression of endogenous BiP, due to stimulated transcription, is a classical result (22). Recently, a similar response has been reported for CRT (39). To our knowledge, however, neither the consequences of these events in terms of Ca\(^{2+}\) accumulation within intracellular stores nor the possibility of modulations in the ER Ca\(^{2+}\) capacity had so far ever been envisaged. Here we show that the capacity of ER stores, calculated on the basis of the \(^{45}\)Ca results, as described previously for CRT-overexpressing cells (13), was considerably increased (68%). Based on the observed increases of both BiP and CRT, as revealed by Western blotting carried out in parallel, and on the ER resting Ca\(^{2+}\) storage levels established by \(^{45}\)Ca experiments with Tg, the two proteins were calculated to participate similarly to the overall increase, contributing \(\sim 25\) and 29\%, respectively, above controls. Thus, the role of these chaperones during stress is not limited to the assistance and quality control of the ER proteins but includes also an increase of the Ca\(^{2+}\) buffering within the
endomembrane system, a process that could contribute to the protection of the cell from the Ca$^{2+}$-dependent damage.

In conclusion, the present results have added a new function to those already recognized for BiP, not only the classical chaperone, but also a Ca$^{2+}$-binding protein playing an important role in the control of the ER luminal Ca$^{2+}$ homeostasis. Although typical not only of BiP but also of other ER proteins, such as CRT and CNX (7, 45), the duality of function does not appear to be the rule since overexpression of another ER lumenal protein, CaBP$_{2}$ (ERp72), remained without appreciable consequences on cell Ca$^{2+}$ homeostasis. This latter negative result with a protein that in vitro is known to bind Ca$^{2+}$ (38, 41) emphasizes the need for Ca$^{2+}$ storage to be investigated also in living cells. At variance with CRT, where Ca$^{2+}$ -binding protein playing an important role in the regulation of the chaperone activity (8, 16, 42) storage also in those other cells. The collaborative assistance and suggestions of Prof. T. Pozzan is gratefully acknowledged. We thank Prof. H. D. Soling for the kind gift of both the plasmid containing the CaBP$_{2}$ (ERp72) cDNA and the corresponding antibody, and Dr. P. Podini for the confocal and electron microscopy.

REFERENCES
1. Bole, D. G., Hendershot, L. M., and Kearney, J. F. (1986) J. Cell Biol. 102, 1558–1566
2. Munro, S., and Pelham, H. R. (1986) Trends Biochem. Sci. 12, 20–23
3. Gething, M. J., and Sambrook, J. (1992) Nature 355, 33–45
4. Vogel, J. P., Misra, L. M., and Rose, M. D. (1990) J. Cell Biol. 110, 1885–1895
5. Hartl, F. U. (1996) J. Cell Biol. 135, 571–580
6. Breuer, J. W., and Hendershot, L. M. (1997) in Molecular Chaperones in Proteins: Structure, Functions and Mode of Action (Fink, A., and Goto, Y., eds) pp. 415–434, Marcel Dekker, New York
7. Gaut, J. R., and Hendershot, L. M. (1993) J. Biol. Chem. 268, 7248–7255
8. Bonifacino, J. S., and Lippincott-Schwartz, J. (1991) Curr. Opin. Cell Biol. 3, 392–400
9. Otsu, M., Urade, R., Kito, M., Omura, F., and Kikuchi, M. (1995) J. Biol. Chem. 270, 14958–14961
10. Werner, E. D., Brodsky, J. L., and Mc Cracken, A. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13797–13801
11. Gaut, J. R., and Hendershot, L. M. (1993) Curr. Opin. Cell Biol. 5, 589–595
12. Bole, D. G., Hendershot, L. M., and Kearney, J. F. (1986) J. Cell Biol. 104, 130, 847–855
13. Montero, M., Brini, M., Marsault, R., Alvarez, J., Sitia, R., Pozzan, T., and Rizzuto, R. (1995) EMBO J. 14, 5467–5475
14. Dorner, A. J., Wasley, L. C., Raney, P., Haugejorden, S., Green, M., and Kaufman, R. J. (1996) J. Biol. Chem. 265, 20209–20214
15. Booth, C., and Koch, G. L. (1989) Cell 59, 729–737
16. Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993) Science 262, 744–746
17. Mery, L., Messelhi, N., Michalak, M., Ops, M., Lew, D. P., and Krause, K. H. (1996) J. Biol. Chem. 271, 9332–9339
18. Dorner, A. J., Wasley, L. C., and Kaufman, R. J. (1992) EMBO J. 11, 1563–1571
19. Bole, D. G., and Hendershot, L. M., Wei, J. Y., Gaut, J. R., Lawson, B., Freiden, P. J., and Muri, K. G. (1995) Mol. Biol. Cell. 6, 283–296
20. Perrin, D., Sonnichsen, B., Soling, H. D., and Nguyen-Van, P. (1991) FEBS Lett. 294, 47–51
21. Villa, A., Podini, P., Panzeri, M. C., Soling, H. D., Volpe, P., and Meddlelsi, J. (1993) J. Cell Biol. 121, 1041–1051
22. Villa, A., Sharp, A. H., Lacchetti, G., Podini, P., Bole, D. G., Dunn, W. A., Pozzan, T., Snyder, S. H., and Meldolesi, J. (1992) Neuroscience 49, 467–477
23. Wada, I., Ou, W. J., Liu, M. C., and Scheele, G. (1994) J. Biol. Chem. 269, 7464–7472
24. Brieri, M., Marsault, R., Bastianutto, C., Alvarez, J., Pozzan, T., and Rizzuto, R. (1995) J. Biol. Chem. 270, 9896–9903
25. Van, N. P., Rupp, K., Lampen, A., and Soling, H. D. (1993) Eur. J. Biochem. 213, 789–795
26. Dorner, A. J., Wasley, L. C., and Kaufman, R. J. (1992) J. Cell Biol. 118, 231–239
27. Dorner, A. J., Wasley, L. C., and Kaufman, R. J. (1996) J. Biol. Chem. 271, 29004–29011
28. Fasolato, C., Zottini, M., Clementi, E., Zacchetti, D., Meldolesi, J., and Pozzan, T. (1995) J. Biol. Chem. 270, 14958–14961
29. Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993) Science 262, 744–746
30. Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993) J. Biol. Chem. 271, 29004–29011
31. Raichman, M., Panzeri, M. C., Clementi, E., Papazafiri, P., Eckley, M., Clegg, D. O., Villa, A., and Meldolesi, J. (1995) J. Cell Biol. 121, 341–354
32. Rooney, E., and Meldolesi, J. (1996) J. Biol. Chem. 271, 29004–29011
33. Fasolato, C., Zottini, M., Clementi, E., Zacchetti, D., Meldolesi, J., and Pozzan, T. (1995) J. Biol. Chem. 270, 20159–20167
34. Michalak, M., Milner, R. E., Burns, K., and Ops, M. (1992) Biochem. J. 285, 681–692
35. Krause, K.-H., and Michalak, M. (1997) Cell 88, 1–11
36. McCormack, J. G., Halestrep, A. P., and Denton, R. M. (1990) Physiol. Rev. 70, 391–485
37. Nigam, S. K., Goldberg, A. L., Ho, S., Robhe, M. F., Bush, K. T., and Sherman, M. Yu. (1994) J. Biol. Chem. 269, 1744–1749
38. Rupp, K., Birnbach, U., Lundstrom, J., Van, P. N., and Soling, H. D. (1994) J. Biol. Chem. 269, 2501–2507
39. Llewellyn, D. H., Kendall, F., Sheik, F. N., and Campbell, K. (1996) Biochem. J. 318, 550–560
40. Macer, D. R. J., and Koch, G. L. E. (1988) J. Cell Sci. 91, 61–70
41. Van, P. N., Peter, F., and Soling, H. D. (1989) J. Biol. Chem. 264, 17494–17501
42. Pozzan, T., Rizzuto, R., Volpe, P., and Meldolesi, J. (1994) Physiol. Rev. 74, 659–685
43. Ting, J., Wunden, S. K., Krix, R., Kelleher, K., Kaufman, R. J., and Lee, A. S. (1987) Gene (Amst.) 55, 147–152
44. Luccero, H. A., Lebeche, D., and Kaminer, B. (1994) J. Biol. Chem. 269, 23112–23119
45. Hebert, D. N., Foellmer, B., and Helenius, A. (1996) EMBO J. 15, 2961–2968