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Association of glucose-regulated protein (grp78) with human keratin 8

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Abstract Keratin polypeptides 8 and 18 (K8/18) are intermediate filament proteins that are expressed in 'simple-type' epithelial cells. They associate with several proteins including the 70 kDa cytoplasmic heat shock proteins (hsp70). We identified the human 78 kDa glucose-regulated protein (grp78) as a keratin-associated protein. Keratin-grp78 association was noted after co-immunoprecipitation of K8/18 from HT29 detergent solubilized cell lysates, and appears to involve non-posttranslationally modified grp78. The grp78-K8/18 association is induced by culturing cells in the presence of tunicamycin or after glucose starvation. K8/18-bound grp78 can be dissociated by Mg-ATP and the association can be reconstituted in vitro using purified grp78, then redissociated again by Mg-ATP. Binding of grp78 occurs preferentially with K8, and when reconstituted does not depend on the posttranslational modification state of K8/18. Co-incubation of K8/18 with hsp70 and grp78 shows preferential association with hsp70. Our results demonstrate a direct association of grp78 with K8 under conditions that induce grp78 expression.

Key words: Keratin; Intermediate filament; Glucose-regulated protein; grp78; Stress protein; hsp70

1. Introduction

Simple-type glandular/secretory epithelial cells express keratin (K) polypeptides 8 and 18 (K8/18) as their major intermediate filament (IF) cytoskeletal protein network, with variable levels of K19 and K20 (reviewed in [1–3]). The keratin family, which is preferentially expressed in epithelial cells, consists of more than 20 members (K1–K20) that form obligate non-covalent heteropolymers of at least one type I (K9–K20) and one type II (K1–K8) keratin. Although the function of IF proteins (including keratins) is still being unraveled, IF posttranslational modifications [4–9] and IF-associated proteins [10,11] are likely candidates for regulating their function. The interaction of keratins with their associated proteins could also regulate the accessibility and/or function of these associated proteins. Several K8/18-associated proteins have been identified including a protein kinase C-ε related kinase [12], an 85 kDa membrane-associated protein [13], the stress-induced and constitutively expressed cytoplasmic heat shock proteins (hsp70) [14,15], and members of the 14-3-3 protein family [16]. In the case of hsp70, association with K8 is noted in asynchronously growing cells (at 37°C) which increases upon heat stress [14]. The hsp70-K8/18 complex dissociates in the presence of Mg-ATP, and can be reconstituted again in vitro by adding purified hsp70 [14]. In contrast to hsp70 binding to K8, 14-3-3 proteins bind to K18 in a keratin phosphorylation-dependent manner [16].

Stress-induced proteins make up a large family whose members are expressed, in a cell compartment-preferential manner, constitutively and/or in an induced fashion (reviewed in [17–21]). Induction of stress proteins occurs in the context of several modalities including heat, glucose starvation, hypoxia, and a variety of toxins. Typically, specific subcellular compartments reflect the distribution of a given stress protein, and some differences are found in the induction mode of the various stress proteins (e.g. heat versus glucose deprivation). For example, the endoplasmic reticulum (ER) contains the 78 kDa (also known as the immunoglobulin binding protein 'BiP', ref. [22–24]) and the 94 kDa glucose-regulated proteins (grp), while the 72 kDa and 73 kDa hsp70 proteins are cytosolic [17,18]. Significant sequence similarity occurs between grp78 and hsp70, as noted for the 61% identity between rat liver grp78 and the 73 kDa rat hsp70 [24]. One of the functions proposed for grp78 is binding to folding polypeptide intermediates to prevent their aggregation. This binding appears to be regulated by grp78 phosphorylation and ADP ribosylation such that non-posttranslationally modified grp78 is the form that is monomeric and available to interact with other proteins [25–27].

During the course of our studies of K8/18-associated proteins, we occasionally noted an association of K8/18 immunoprecipitates with a 78 kDa protein. Purification of this protein followed by tryptic digestion then microsequencing identified a peptide that was homologous with the 78 kDa glucose-regulated protein grp78. Grp78 is known to be induced in cells under a variety of conditions including glucose starvation and culturing in the presence of tunicamycin or the calcium ionophore A23187 [19,20], and this induction correlated with grp78 association with K8/18 immunoprecipitates. We also show that grp78 associates with K8/18 in an ATP-dependent fashion, and that the association occurs preferentially with K8 rather than with K18.

2. Materials and methods

2.1. Cells and reagents

HT29 cells were obtained from the American Type Culture Collection (Rockville, MD). Antibodies used were: monoclonal antibody (mAb) L2A1 [28], anti-human K8/18 rabbit antibody 8592 [16], rabbit anti-human grp78 (StressGen; Victoria, BC, Canada). Other reagents used were: okadaic acid (LC Services; Woburn, MA), tunicamycin (Tn) and hsp70 (Sigma; St. Louis, MO), polyvinylidene difluoride (PVDF) membrane (Millipore; Bedford, MA), immobilized protein

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Abbreviations: Emp, Empigen BB; ER, endoplasmic reticulum; IF, intermediate filament(s); K, keratin; mAb, monoclonal antibody; NP40, Nonidet P40; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride

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A (Pierce Chemical; Rockford, IL), enhanced chemiluminescence (ECL) reagent (Amersham; Arlington Heights, IL).

2.2. Cell culture

HT29 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C. Enrichment for mitotic cells was done by culturing cells with aphidicolin (5 μg/ml, 24 h) followed by washing off the aphidicolin then culturing in normal growth media for 10 h [29]. For heat stress, cells (~50% confluent) were cultured at 42°C for 24 h. Induction of grp78 was done by culturing cells in the presence of tunicamycin (5 μg/ml, 37°C) for 16 h, or culturing in glucose-free medium for 16 h.

2.3. Gel analysis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% polyacrylamide gels under non-reducing conditions [30]. Proteins in the gels were stained with Coomassie brilliant blue R-250. Two-dimensional gel electrophoresis was done using isoelectric focusing then SDS-PAGE. For immunoblotting, proteins from SDS-PAGE gels were transferred to PVDF membranes. After transfer, membranes were rinsed with 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl (PBS). Blotting was done as described [29], and reactive proteins were visualized using an ECL system as recommended by the manufacturer.

2.4. Immunoprecipitation

Cells were collected, washed with PBS/5 mM EDTA, then solubilized with 1% Nonidet P-40 (NP40) or 1% Empigen BB [31] in PBS (pH 7.4) containing 5 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 10 μM pepstatin A, 10 μM leupeptin, 25 μg/ml aprotinin and 0.5 μg/ml okadaic acid (45 min, 4°C). After spinning (13 000×*g*, 30 min, 4°C), supernatants were incubated with mAb L2A1 (covalently conjugated to protein A-agarose) for 6 h (4°C) followed by washing off unbound material.

2.5. Release of grp78/hsp70 from K8/18 immunoprecipitates and reconstitution of K8/18-grp78 and hsp70 binding

K8/18 immunoprecipitates were obtained from 1% NP40 detergent lysates of HT29 cells that were cultured at 37°C in the presence of tunicamycin (5 μM, 16 h). In order to release K8/18-associated grp78/hsp70, precipitates were incubated for 1 h (22°C) with 5 mM Na-ATP plus 5 mM $MgCl_2$ (Mg-ATP) then washed. Alternatively, immunoprecipitates were first treated with Mg-ATP, washed three times to remove released grp78/hsp70, then incubated with 50 μl of PBS containing 1 μg of recombinant hamster grp78 (StressGen), or bovine brain hsp70, or a mixture (1 h, 22°C). After washing, the binding of grp78 and/or hsp70 to K8/18 was analyzed by SDS-PAGE.

2.6. Binding of grp78 to K8 versus K18

K8 and K18 were purified individually from HT29 cells. For this, K8/18 immunoprecipitates were prepared using mAb L2A1, followed by preparative SDS-PAGE to separate K8 from K18, brief staining with Coomassie blue, cutting out individual K8 and K18 bands, then electroelution. Eluted K8 and K18 were then precipitated using 100% methanol (−20°C), air dried, solubilized in 9 M urea, then dialyzed against 1% Empigen in PBS/2 mM EDTA to allow renaturation. The renaturated K8 and K18 were individually reimmunoprecipitated using anti-K8/18 antibody 8592 (conjugated to protein A-agarose) followed by incubation with grp78 then washing excess unbound grp78.

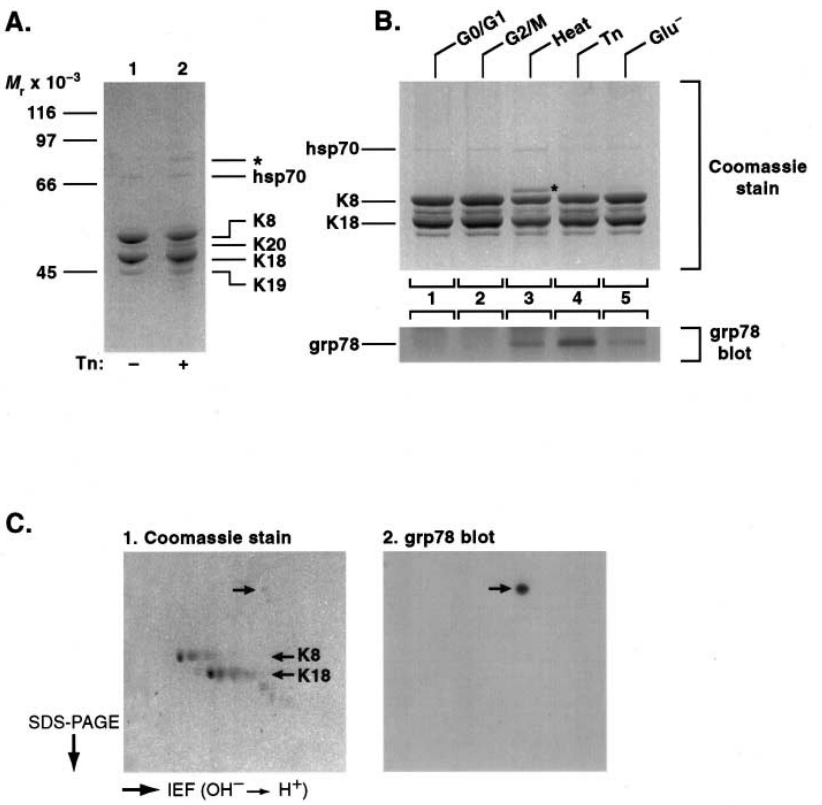

Fig. 1. K8/18 bind to grp78. Panel A: HT29 cells were cultured in the presence or absence of tunicamycin (Tn) (5 μg/ml, 16 h). K8/18 immunoprecipitates were subsequently obtained followed by SDS-PAGE analysis then Coomassie staining. Asterisk in lane 2 indicates the 78 kDa protein that associates with K8/18 in Tn-treated cells. Panel B: Immunoprecipitates of K8/18 were obtained from asynchronously growing HT29 cells (primarily G0/G1 stage of the cell cycle) (lane 1), G2/M cell cycle stage enriched cells (lane 2), cells that were cultured at 42°C for 24 h (lane 3), cells that are cultured with Tn (5 μg/ml, 16 h) (lane 4), or cells cultured in glucose-free media for 16 h (lane 5), followed by analysis on duplicate SDS-PAGE gels. One gel was stained with Coomassie blue and the duplicate was transferred to a PVDF membrane followed by blotting with anti-grp78. Panel C: A K8/18 immunoprecipitate that is identical to that shown in panel A, lane 2, was analyzed by isoelectric focusing then SDS-PAGE. The faint Coomassie stained band that is highlighted by the unlabeled arrow (histogram #1, pI ~5) corresponds to grp78 as determined by immunoblotting (also indicated by an unlabeled arrow in histogram #2).

3. Results

3.1. Identification of grp78 as a K8/18 binding protein

During our studies of K8/18-associated proteins, we sometimes noted an association of a 78 kDa protein which was distinct from the already characterized keratin association with hsp70 [14,15]. Purification of this associated protein followed by microsequencing of a tryptic fragment showed 100% identity of the fragment with grp78 (not shown). This led us to examine the association of the 78 kDa protein with K8/18 that was isolated from Tn-treated cells. As shown in Fig. 1A, treatment of human colonic HT29 cells with Tn resulted in co-immunoprecipitation of a 78 kDa protein (indicated by an asterisk) with K8/18, which was barely seen in non-Tn-treated cells. This correlated with increased expression of grp78 (not shown), which has been described upon Tn treatment and glucose starvation in a variety of cell systems [19,20]. Further confirmation that the 78 kDa keratin-associated protein corresponds to grp78 was obtained by immunoblotting of K8/18 precipitates, that were obtained from several cell culture conditions. As shown in Fig. 1B, grp78 association with K8/18 was easily detectable upon Tn treatment, glucose starvation, or heat stress. Immunoblotting of a K8/18 precipitate that was obtained from Tn-treated cells then analyzed by two-dimensional gels showed that the 78 kDa K8/18-associated protein migrated as a single isoform when immunoblotted with anti-grp78 (Fig. 1C). In addition, in vivo $^{32}PO_4$-labeling showed lack of ^{32}P-phosphate incorporation into the K8/18-associated grp78 (not shown). This suggests that the grp78 that binds K8/18 is the non-phosphorylated, non-ADP-ribosylated form of grp78 as has been shown previously for other grp78 binding proteins (e.g. [27]).

3.2. Dissociation of the K8/18-grp78 complex by Mg-ATP, and relative binding of hsp70 versus grp78 to K8/18

As previously noted for K8/18-hsp70 [14], the K8/18-grp78 (and hsp70) complex also dissociates in the presence of Mg-ATP (Fig. 2A, compare lanes 1 and 2). Binding of K8/18 to

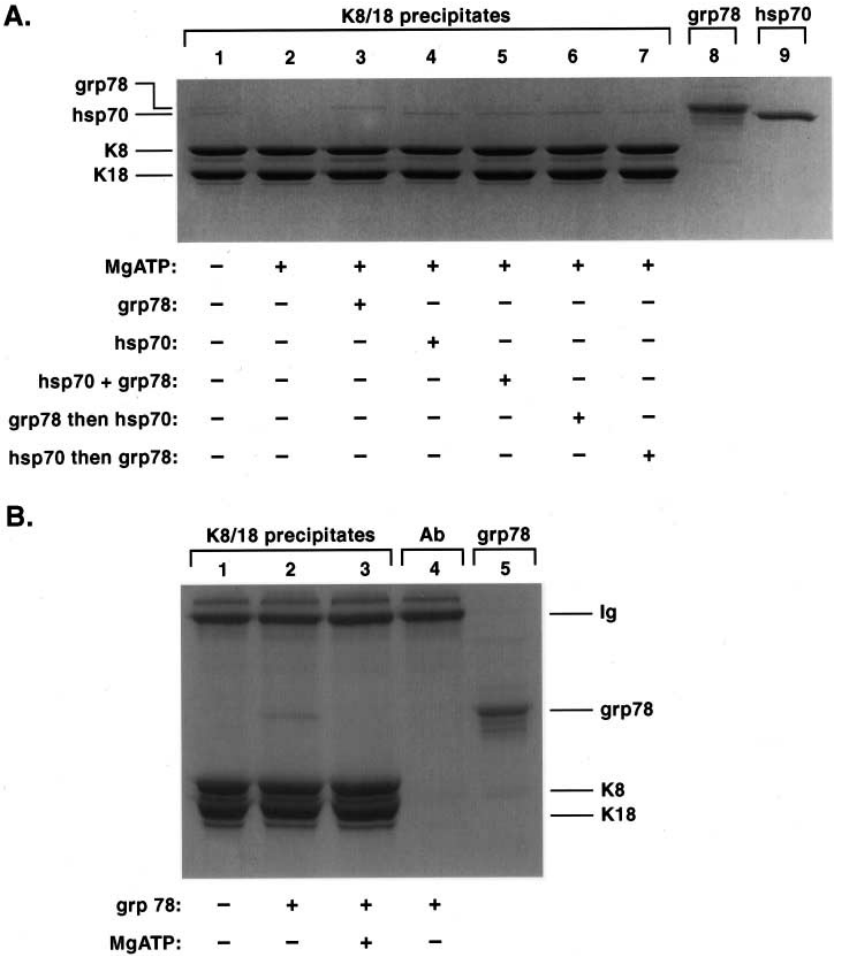

Fig. 2. Release of grp78 by Mg-ATP and comparison of K8/18 binding to hsp70 and grp78. Panel A: K8/18 immunoprecipitates were obtained from Tn-treated HT29 cells (lane 1), followed by incubation with 5 mM Mg-ATP (1 h, 22°C) (lane 2) to release hsp70 and grp78. After washing, Mg-ATP-treated precipitates (in duplicate) were incubated with 1 μg (in 100 μl of PBS) of grp78 (lane 3) or hsp70 (lane 4) (1 h, 22°C) followed by washing three times to remove unbound protein. The recombinant grp78 and hsp70 (shown in lanes 8 and 9, respectively) were then added to the identical set of precipitates shown in lanes 4, 5 such that 1 μg of hsp70 was added to the grp reconstituted complex (lane 6), or 1 μg of grp78 was added to the hsp70 reconstituted complex (lane 7). After washing, samples were analyzed by SDS-PAGE. Panel B: K8/18 immunoprecipitates were obtained from Tn-treated HT29 cells, then incubated with 5 mM Mg-ATP (1 h, 22°C) followed by washing. Duplicates of the hsp70/grp78-depleted precipitate (lane 1) were incubated with grp78 (1.5 mg, 60 min, 22°C) followed by washing (lane 2), then incubated with 5 mM Mg-ATP (1 h, 22°C) (lane 3). Similarly, antibody-Sepharose beads were incubated with grp78 without any significant evidence of binding (lane 4). The input grp78 is shown in lane 5.

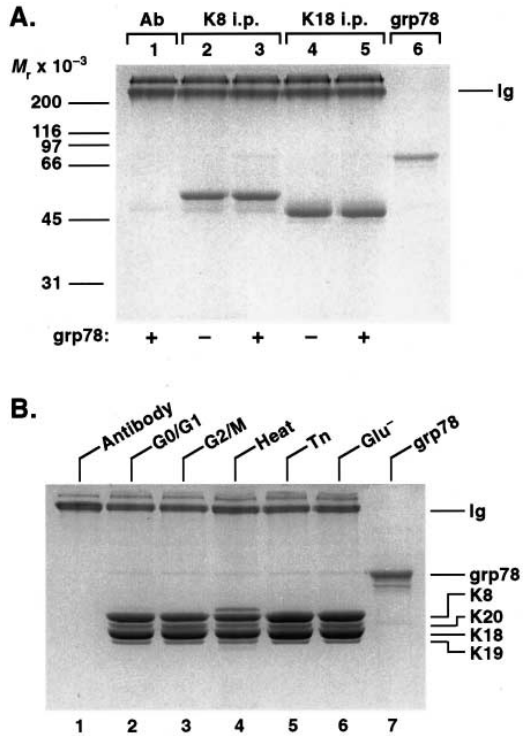

Fig. 3. Grp78 binds to K8, in vitro, independent of keratin post-translational modifications. Panel A: K8 and K18 were individually purified then immunoprecipitated as described in Section 2. Grp78 was added to the K8 (lane 3), K18 (lane 5), or to an antibody control immunoprecipitate (lane 1). Panel B: K8/18 immunoprecipitates were obtained from HT29 cells (G0/G1, G2/M, heat stressed, Tn-treated, and glucose starved as in Fig. 1B). The precipitates were incubated with Mg-ATP to release endogenously bound hsp70/grp78 followed by washing. Precipitates were then incubated with grp78 followed by washing then analysis by SDS-PAGE and Coomassie staining.

grp78 and hsp70 can be reconstituted in vitro by incubating 'stripped' K8/18 precipitates (i.e. those shown in Fig. 2A, lane 2) with grp78 (lane 3) or hsp70 (lane 4). Incubation of 'stripped' K8/18 precipitates with a mix of hsp70+grp78 showed a preferential binding of K8/18 with hsp70 (Fig. 2A, lane 5). Furthermore, hsp70 appears to compete out K8/18-bound grp78 when added in the sequence grp78-then-hsp70, while grp78 does not compete out K8/18-bound hsp70 when added in the sequence hsp70-then-grp78 (Fig. 2A, lanes 6 and 7, respectively).

The in vitro release of grp78 from a K8/18-grp78 complex was also observed subsequent to the in vitro reconstitution of K8/18-grp78 association. Hence, as shown in Fig. 2B, incubation of grp78-stripped K8/18 precipitates (shown in lane 1) with grp78 reconstitutes the binding (lane 2) which in turn can be stripped again by reincubating with Mg-ATP (lane 3). Of note, grp78 does not bind to the anti-K8/18 antibody that was used for immunoprecipitation, thereby supporting the specificity of the K8/18-grp78 interaction.

3.3. Binding of grp78 to K8/18 involves interaction with K8 and is independent of the posttranslational state of K8/18

We previously showed that hsp70 binds to K8 rather than K18 [14]. The preferential binding of hsp70 to K8/18 when added together with grp78 (Fig. 2A) suggests that grp78 may also bind to K8. We tested this formally by incubating K8 or K18 precipitates with grp78 followed by washing off unbound protein. As shown in Fig. 3A, grp78 associates preferentially and specifically with K8 rather than K18.

We also examined in vitro if changes in K8/18, upon Tn treatment or glucose starvation, accounted for the observed K8/18-grp78 binding under these conditions. As shown in Fig. 3B, reconstitution of grp78-K8/18 binding, using K8/18 precipitates (stripped) that were obtained from cells grown under a variety of conditions, showed similar levels of binding to grp78 in a manner that did not appear to be affected by the source of the K8/18. This suggests that the promotion of grp78-K8/18 binding seems to be at the level of grp78 rather than K8/18.

4. Discussion

This report demonstrates binding of K8/18 immunoprecipitates with grp78. Binding of grp78 to the K8/18 heteropolymer occurs preferentially with K8, and the complex is released in the presence of Mg-ATP. The grp78-K8/18 association is similar to the previously described K8/18-hsp70 association [14] in that: (i) it occurs with K8, (ii) dissociation of the complex is induced in the presence of Mg-ATP, and (iii) conditions that increase hsp70 (e.g. heat stress) or grp78 (e.g. culture in the presence of Tn or in the absence of glucose) levels also increase keratin association with the corresponding protein. Of note, Tn treatment does not increase hsp70-keratin association (not shown) and heat stress increases grp78-K8/18 association only slightly. Although the precise location(s) of K8 binding to grp78 and hsp70 remains to be defined, the ability of hsp70 to preferentially bind to K8 in the presence of grp78 suggests the K8 binding site(s) to hsp70 and grp78 is (are) likely to be overlapping if not identical, and that hsp70 has a higher affinity than grp78 to K8.

The K8/18-bound grp78 corresponds to the major isoelectric isoform which in turn likely corresponds to the non-phosphorylated, non-ADP-ribosylated form of grp78. Several previous studies showed that it is this non-posttranslationally modified form of grp78 that binds to its ligands [25–27]. Our data also indicate that recombinant grp78 binding to K8/18 does not depend on the posttranslational modification state of the keratin. For example, heat stress and mitosis induce significant phosphorylation of K8 ser73 [32], K8 ser431 [33], and K18 ser52 [29]. In addition, heat stress increases K8/18 glycosylation [15]. Yet keratins that were isolated from mitotic or heat stressed cells bound similar amounts of recombinant grp78 as keratins that were isolated from interphase or Tn-treated cells.

Interaction of a grp78-like protein with a testis-specific serine/threonine phosphatase PP1γ2 has also been described, and in this case the 78 kDa protein was detected in spermatocyte and spermatid nuclei [34]. Also, a T-lymphocyte cell surface protein that is related to grp78 was recently described [35]. The 'ectopic' presence of normally ER-resident proteins may occur by masking of the ER retention signal as proposed for the presence of calnexin on the surface of immature thymocites [36]. In addition, interaction of the related grp94 with actin has been described [37]. Although the K8/18 interacting 78 kDa protein in cultured cells may be related to, rather than identical with, grp78, the latter possibility is more likely. This is based on the sequence homology of the isolated tryptic fragment, binding with the anti-grp78 antibody, migration

on SDS-PAGE and isoelectric focusing gels, reconstitution of the binding with purified grp78, and release of the complex by Mg-ATP.

The physiologic relevance of K8/18-grp78 binding remains to be determined. The location of grp78 in the endoplasmic reticulum (ER) makes any association of K8/18 with grp78 significant only under conditions of ER damage, or export of resident lumenal ER proteins into the cytosol or the cytoplasmic resident soluble keratins into the ER. In the case of HT29 cells, only a minor fraction of the total grp78 cellular pool (and the keratin-associated grp78) is present in the cytosol (after cell disruption, not shown). Therefore, any potential physiologic association of grp78 with K8 is likely to occur primarily in conditions that result in ER damage and subsequent leakage of soluble ER contents into the cytosol.

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