Peptidoglycan Recognition Protein Tag7 Forms a Cytotoxic Complex with Heat Shock Protein 70 in Solution and in Lymphocytes*

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The peptidoglycan recognition protein Tag7 is shown to form a stable 1:1 complex with the major stress protein Hsp70. Neither protein is cytotoxic by itself, but their complex induces apoptotic death in several tumor-derived cell lines even at subnanomolar concentrations. The minimal part of Hsp70 needed to evoke cytotoxicity is residues 450–463 of its peptide-binding domain, but full cytotoxicity requires its ATPase activity; remarkably, Tag7 liberated from the complex at high ATP is not cytotoxic. The Tag7-Hsp70 complex is produced by tag7-transfected cells and by lymphokine-activated killers, being assembled within the cell and released into the medium through the Golgi apparatus by a mechanism different from the commonly known granule exocytosis. Thus, we demonstrate how a heat shock protein may perform functions clearly distinct from chaperoning or cell rescue and how peptidoglycan recognition proteins may be involved in innate immunity and anti-cancer defense.

The tag7/PGRP* gene, first found in mice (1, 2) and somewhat later in insects (3), belongs to a novel family of conserved genes (including four human members) that encode peptidoglycan recognition proteins (3–6) with structural similarity to T-phase lysozyme in the C-proximal domain (3). Recombinant PGRP and natural bovine PGRP have been reported to be bacteriostatic and bactericidal (3, 7). However, recombinant PGRP produced in a baculovirus system had no cytotoxic effect (8), whereas we did observe Tag7-associated cytotoxicity. The Tag7-Hsp70 complex is produced by tag7-transfected cells and by lymphokine-activated killers, being assembled within the cell and released into the medium through the Golgi apparatus by a mechanism different from the commonly known granule exocytosis. Thus, we demonstrate how a heat shock protein may perform functions clearly distinct from chaperoning or cell rescue and how peptidoglycan recognition proteins may be involved in innate immunity and anti-cancer defense.

Our previous studies have shown the following: (i) that lymphokine-activated killers exert their cytotoxicity through secretion as well as membrane-associated proteins (9, 10); (ii) that the secretory mechanism of cell lysis is not restricted to exocytosis of granzymes: e.g. cytotoxic proteins are released in the absence of calcium (11); and (iii) that the action of the latter can be largely blocked with anti-Tag7 antibodies (12). Hence, we make the suggestion that Tag7 initiates the cytotoxic events not by itself but in complex with some other protein(s).

Searching for the Tag7 partner, here we show that in vitro Tag7 readily forms a complex with Hsp70. The latter is a multifunctional protein involved in protein translocation, folding, and multimer assembly (13) as an ATP-dependent molecular chaperone; again, Hsp70 has recently been reported to prevent apoptosis (14, 15) and to act outside as well as within the cell (16). Further, we show that the complex of purified recombinant Tag7 and Hsp70 exerts marked cytotoxic activity at subnanomolar concentrations and that human lymphokine-activated killers enriched in CD3 and CD8 markers do use the Tag7-Hsp70 complex as a cytotoxic agent released through the Golgi apparatus.

EXPERIMENTAL PROCEDURES

Cells—Mouse L929 fibroblasts, SV40-transformed African green monkey kidney COS-1, baby hamster kidney BHK21, human erythroblasts K562, human T-lymphoma Jurkat, mouse adenocarcinoma C32-ML-0, and human melanoma M3 cells were cultured in RPMI 1640 supplemented with 2 mm t-glutamine and 10% fetal calf serum (all from Invitrogen). The Tag7 cDNA was subcloned in pBK-CMV (Stratagene) and pEGFP (Clontech) vectors. For Tag7 or Tag7-EGFP expression, COS-1 or BHK21 cells were transiently transfected with the corresponding constructs using MaxiFectin (Qiagen) reagents according to the manufacturer’s recommendations, and the cells were used in 24 h.

To prepare lymphokine-activated killers, human peripheral blood was obtained from healthy volunteers at the Cancer Research Center (Moscow); mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation and cultivated for 6 days with 1000 units/ml of recombinant interleukin-2 (Sigma), granule lymphocytes were isolated by Percoll gradient centrifugation (9).

Proteins and Antibodies—The cDNAs for recombinant mouse Tag7, mouse metastasin (Mts1)/S100A4, human 70-kDa heat shock protein 1A (Hsp70), and Hsp70 peptide-binding domain (385–539) were subcloned in pQE-31 and expressed in E. coli. The proteins were purified on nickel nitritoltriacetic acid-agarose (Qiagen) as recommended by the manufacturer. Mouse rTag7 produced in yeast was kindly provided by Dr. S.V. Benevolensky. The p70 protein was isolated from L929 cell membranes as described elsewhere (17) and used to raise polyclonal antibodies in mice.

Rabbit antibodies raised against recombinant Tag7 or Hsp70 were affinity-purified on CNBr-activated Sepharose 4B (Amersham Biosciences) coupled with rTag7 and rHsp70 in accordance with the manufacturer's manual. Rabbit antibodies against human TNFα were from...
Sigma, and 3A3 mouse monoclonal anti-Hsp70 was from Affinity Bioreagents Inc. 3B5 mouse monoclonal anti-Hsp70 was a gift of Dr. I. V. Guzhova. Mouse monoclonal anti-GM130 was from Transduction Laboratories, Lexington, KY. 3C1 rat monoclonal anti-rTag7 was produced by a standard hybridoma protocol (18), and clone 3CIB6 antibodies recognized both native and recombinant Tag7.

Unless specified otherwise, the standard incubation time for protein complexing was 37 °C in PBS pH 7. Cell membranes were isolated as described (19), and, after removing lipids with ether, membrane protein was suspended in PBS with a protease inhibitor mixture (Roche Diagnostics) to 3–5 mg/ml. To obtain secreted proteins, lymphocytes, after 6 days of activation with 1000 units/ml rIL-2, were incubated with either K562 cells (100:1) for 18 h in serum-free RPMI 1640 or with phorbol myristate acetate (20 ng/ml) and ionophore A23187 (200 nM); COS-1 cells 24 h after transfection were cultivated for 18 h in serum-free RPMI 1640. Cells were removed by centrifugation; the conditioned medium was tested for the presence of Tag7 or Tag7-EGFP by immunoblotting, and the supernatants were used in cytotoxic assays.

Affinity Chromatography, Immunoadsorption, and Immunoblotting—Tag7 produced in E. coli was coupled to CNBr-activated Sepharose 4B (Amersham Biosciences) according to the manufacturer’s instructions. The proteins of the membrane fractions from L929 cells were loaded onto a Tag7-Sepharose 4B column, washed with PBS and 0.5 M NaCl, eluted with 0.25 M triethylamine, concentrated, resolved by 12% PAGE with SDS, and stained with Coomassie Brilliant Blue R250 (Serva).

Purified antibodies were also coupled to CNBr-activated Sepharose 4B. The Tag7-Hsp70 complex, the conditioned medium of COS-1-tag7 cells, and the conditioned medium of lymphokine-activated killers were loaded on antibody-conjugated Sepharose. Then the material was extensively washed with PBS plus 0.5 M NaCl followed by PBS, eluted with 0.25 M triethylamine, pH 12, or 50 mM ATP, concentrated, and resolved by 12% PAGE with SDS, and stained with Coomassie Brilliant Blue R250 (Serva).

The p70 protein of the L929 cell membranes was purified by SDS-PAGE, the corresponding gel region was excised and extracted with acetone, and the protein was digested with trypsin (Sigma) by a standard protocol (Ciphergen Biosystems, Inc.). The tryptic digest of p70 was analyzed by MALDI-TOF mass spectrometry (23), and selected mass values from the spectra were used to search the Swiss-Prot protein database.

RESULTS

Tag7 Binds with Hsp70—To test the idea that Tag7 acquires cytotoxic activity upon forming a complex with another protein, solubilized membrane proteins from target L929 cells were subjected to affinity chromatography on Tag7-conjugated Sepharose. The specifically bound protein was eluted as a single peak of 70 kDa. A similar p70 was found in several other cell lines.

The obvious next step was to establish the nature of this potential partner. By mass spectrometric analysis of tryptic peptides, the Tag7-binding p70 was found to be identical to the heat shock protein Hsp70. Recombinant Hsp70 was also shown to be bound specifically on Tag7-Sepharose. The identities of the isolated p70 and the rHsp70 were proven by Western blotting with mouse antibodies to p70 and rabbit antibodies to Hsp70, which were fully cross-reactive (Fig. 1A).

To directly demonstrate the interaction, recombinant Tag7 and Hsp70 were mixed (10 nM each) and then immunoprecipitated with Sepharose-coupled antibodies against Tag7 or Hsp70. In either case, the immune complex contained both proteins (Fig. 1B), whereas control incubations with individual antibodies showed that there was no cross-reactivity. Hence, one can state that Tag7 and Hsp70 form a specific complex in solution.

Complexing in vitro—To determine the stoichiometry of the Tag7-Hsp70 complex, the two proteins and their mixture were subjected to gel filtration. Preincubations and HPLC runs were carried out in the presence of 5 μM ATP to avoid interference from Hsp70 self-association (24). (Indeed, 200–4000 kDa aggregates were formed by Hsp70 alone and in mixture with Tag7 in preliminary experiments without ATP.) Fig. 2A shows that, under such conditions, Hsp70 alone behaved as a 70-kDa en-
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proteins form a 1:1 complex.

EGFP (50 kDa) constructed for subsequent

in situ

activity, and Tag7 alone acted as a 20-kDa entity, whereas in their mixture most of the protein was eluted in the 90-kDa peak. The latter contained both Tag7 and Hsp70, as evidenced by Western blotting (Fig. 2B). These data strongly suggest that the two proteins form a 1:1 complex.

Further, similar analysis was performed with a fusion Tag7-EGFP (50 kDa) constructed for subsequent in situ fluorescence analysis; HPLC demonstrated formation of a 120-kDa fluorescent complex with Hsp70 (Fig. 2C), thus confirming the 1:1 stoichiometry as well as proving the adequacy of the labeled construct. EGFP itself did not interact with Hsp70 (not shown).

The association process was found to be markedly ATP-dependent. Very little of the 90-kDa complex was formed by purified proteins in the absence of ATP; a moderate molar excess (5 μM ATP at 1 μM each protein) ensured fast complexing, whereas 50 mM caused the complex to dissociate. If the complex was adsorbed on anti-Tag7 or anti-Hsp70-Sepharose and then treated with 50 mM ATP, Hsp70 was eluted in the former case and Tag7 in the latter (lane 1 in Fig. 3).

The Tag7-Hsp70 Complex Induces Cell Death in Culture—Next, we checked whether this newly found complex could indeed be cytotoxic. As shown in Fig. 4A, Tag7-Hsp70 at 1 nM caused the death of about one-third of L929 cells in 24 h, although either protein added separately had no appreciable effect. The cytotoxic action of the complex was prevented by the simultaneous addition of purified polyclonal antibodies against either rTag7 or rHsp70 (but not by immunoglobulins from nonimmunized animals). As above, all incubation media included 5 μM ATP; without it, the cytotoxic effect was considerably lower and less reproducible.

The concentration dependence of cytotoxicity assessed with equimolar mixtures of Tag7 and Hsp70 (Fig. 4B) shows that the maximal effect was already reached at 0.1 nM, corroborating the specificity of the phenomenon. The L929 cells exposed to Tag7-Hsp70 died by an apoptotic pathway as evidenced by increased DNA fragmentation (Fig. 4C); the profiles obtained with either protein alone did not differ from the control. We also checked the effect of Tag7-Hsp70 on some other cell lines: tumor-derived Jurkat, M3, and CSML-0 were susceptible, whereas human lymphocytes and fibroblasts from primary cell culture were not.

Role of Hsp70 Domains in Functional Interaction with Tag7—Further, we tried to determine some structural requirements for the cytotoxic activity. Hsp70 has two domains of known function, ATP binding and peptide binding (24). Cytotoxicity was evoked by the incubation of Tag7 with the isolated peptide-binding domain (Hsp70 amino acids 385–539) and its tetracapeptide 450–463 (TKDNLLGRFELSG), considered crucial for target protein binding (25), whereas the corresponding Hsc70 tetrapeptide, which has two changes, R458K and S462T, was totally ineffective (Fig. 4B). However, with Hsp fragments, an appreciable cytotoxic effect was attained only at concentrations three orders of magnitude higher than that of the full-sized protein. Together with the ATP requirement, this means that the ATP-binding moiety is also quite important.

ATP Hydrolysis Is Necessary for Tag7-Hsp70 Cytotoxicity—In the conventional chaperone cycle, ATP hydrolysis in the active center of Hsp70 is needed for the bound target protein to assume the proper conformation, whereas the reaction product, ADP, stabilizes the complex; further replacement of ADP with ATP leads to its dissociation (26–28).

We found (Fig. 4D) that the ATPase activity of Hsp70 increased significantly upon complexing with Tag7, which itself was inactive in this respect. Cytochrome c, used as a control, was without effect. The positive control was metastasin (Mts1 or S100A4), which, as we have shown previously, interacts with the plasma membrane calcium-ATPase (29); its effect on Hsp70 was close to that of Tag7.

The Hsp70-Mts1 complex was not cytotoxic. Interestingly,
Fig. 4. Features of the Tag7-Hsp70 complex. A, the Tag7-Hsp70 complex is cytotoxic, whereas its constituents are not. rTag7 and rHsp70 (1 nM each) were mixed in PBS with 5 µM ATP, incubated for 1 h at 37 °C, and added to L929 cells. Cell death was assayed as described under “Experimental Procedures.” The plots give the percentage of specifically killed cells (i.e. minus spontaneous death, which did not exceed 8%). Rabbit polyclonal anti-Tag7 or anti-Hsp70 antibodies (1:500) were added simultaneously with the complex. B, concentration dependences of cytotoxicity produced by equimolar combinations of Tag7 with full-sized Hsp70 (with 5 µM ATP (line 1) and without ATP (line 2)) and heat shock protein fragments in the absence of ATP-Hsp70 peptide binding domain (line 3), 14-mer Hsp70 peptide (line 4), and 14-mer Hsc70 peptide (line 5). Assay was performed as described for panel A. C, Tag7-Hsp70 induced apoptosis in L929 cells. Tag7 and Hsp70 (0.1 nM each) were incubated with 5 µM ATP for 1 h at 37 °C and added to L929 cells. After 24 h, the cells were analyzed by flow cytometry, and the DNA content was assessed from the fluorescence intensity. The diagram indicates the percentage of apoptotic cells, i.e. those with fragmented DNA. D, ATPase activity of Hsp70 is enhanced by Tag7 and Mts1. ATP hydrolysis was measured in 20 mM KCl, 1 mM EGTA, 10 mM Na₂SO₃, 2 mM ATP, 4 mM MgSO₄, and 20 mM Tris-HCl, pH 8.2 at 37 °C for 15 min. The proteins (Hsp70, Tag7-Hsp70 complex, Tag7, cytochrome c, and Mts1) were added at 1.5 nM. E, addition of 5 µM AMP-PNP, a nonhydrolyzable ATP analog, completely abolishes Tag7-Hsp70 cytotoxicity. Hsp70 with equimolar Mts1 is not cytotoxic. Mts1 competes with Tag7 for Hsp70 binding to form a nontoxic complex but does not affect the already existing Tag7-Hsp70 complex. Eluted Tag7 (i.e. rTag7 dissociated from the complex at 50 nM ATP (Fig. 3) does not acquire cytotoxic activity. The assay was performed as described for panel A.
an empty vector (cells were transiently transfected with a tag7-expressing construct or an empty vector (neo). Conditioned medium (Sn, ~100 ng of total protein) or membrane proteins (Mb, 50 ng) were added to L929 cells. Cell death was determined after 24 h at 37°C. Polyclonal antibodies to Tag7 or Hsp70 (1:500) were added to the conditioned medium and to the membrane proteins. B, complex of 90 kDa detected in the membranes of COS-1-tag7 cells (lanes 1) and cytotoxic lymphocytes (lanes 2) by Western blotting with 3C1 and 3A5 antibodies to Tag7 and Hsp70, respectively. The proteins were resolved by PAGE with SDS but without β-mercaptoethanol.

the simultaneous addition of Mts1 and Tag7 to Hsp70 hindered the formation of a cytotoxic Tag7-Hsp70 complex, as Mts1 successfully competed with Tag7 for Hsp70. However, a 10-fold excess of Mts1 added after Tag7 could not interfere with Tag7-Hsp70 cytotoxicity (Fig. 4E; see also the subsection “Tag7-Hsp70 Is Released by Lymphokine-activated Killers”).

In this context, we checked whether ATP cleavage in Tag7-Hsp70 was essential to its cytotoxicity (Fig. 4E). Indeed, the complex failed to kill target cells in the presence of 5 µM AMP-PNP, a nonhydrolyzable ATP analog. Notably, Tag7 liberated from the complex by ATP elution again had no cytotoxic effect (rightmost bar in Fig. 4E); i.e. stable complexing with Hsp70 rather than mere correct folding was required to induce cell death.

Detection of Tag7-Hsp70 in tag7-Transfected Cells—Proceeding to the in vivo level, we first had to check whether such a complex could indeed be produced by a live cell, and we thus started with transfecting standard laboratory lines to express tag7. In line with earlier studies (2), the conditioned medium of COS-1-tag7 cells exhibited cytotoxicity that could be suppressed with antibodies to Tag7 as well as to Hsp70 (Fig. 5A). Just as in the in vitro experiments described above, the cytotoxic component was immunoadsorbed by anti-Tag7 and anti-Hsp70-Sepharose; elution with 50 mM ATP liberated Hsp70 and Tag7, respectively (Fig. 3, lane 2), confirming the presence of a Tag7-Hsp70 complex.

To check whether the complex was formed already within the cell, similar assays were run on the membrane fraction from the transfected COS-1 cells, yielding the same results (Fig. 5A). Further, nonreducing SDS-PAGE of membrane proteins bound to anti-Tag7-Sepharose revealed a 90-kDa band reacting with both antibodies (Fig. 5B).

To locate the Tag7-Hsp70 complex in the cell, we used confocal microscopy of BHK cells transfected with the tag7-EGFP construct and stained with anti-Hsp70 and anti-Golgi antibodies (Fig. 6). Though the distribution of Hsp70 was expectedly rather diffuse, its co-localization with the bulk of Tag7 fluorescent fusion in the Golgi apparatus is quite obvious.

Tag7-Hsp70 Is Released by Lymphokine-activated Killers—Having shown that a cytotoxic Tag7-Hsp70 complex can arise in solution and in transfected cells, we had to demonstrate this phenomenon in a natural system to speak about its biological relevance. To this end, human lymphocytes activated with interleukin-2 for 6 days and enriched in cytotoxic T-lymphocyte markers CD8 and CD3+ (further referred to as LAKs) were co-incubated with K562 cells (100:1). Their conditioned medium killed some 28% of L929 cells in our standard assay (it should be noted that the latter proved quite selective for the events under study: indeed, L929 cells are devoid of the Fas receptor, and granzymes have little effect without perforin, which is apparently not produced by these LAKs; Refs. 11, 12). Cell death could be prevented with polyclonal antibodies against Tag7 or Hsp70, but not those against TNF receptor (Fig. 7A); the antibodies specifically recognized the agents released by LAKs, as evident from the calculated dissociation constants (5.3 × 10⁻¹¹ M for anti-Tag7 and 2.5 × 10⁻¹¹ M for anti-Hsp70). The addition of 5 µM ATP to the assay medium raised the cytotoxicity to 35%, whereas 5 µM AMP-PNP completely blocked it (Fig. 7A), just as with Tag7-Hsp70 in vitro.

The LAK-conditioned medium was passed through anti-Tag7 or anti-Hsp70-Sepharose; elution with 50 mM ATP yielded Hsp70 in the former case and Tag7 in the latter (Fig. 3, lane 3). All these data indicated that CD8+ and CD3+ lymphocytes released the Tag7-Hsp70 complex in response to contact with target cells. To completely rule out the possibility that the cytotoxic proteins were secreted by the K562 cells (even if unlikely in view of their minority), LAKs were instead induced with phorbol myristate acetate and ionophore A23187 (31), with quite the same results (not shown).

To ascertain whether the complex was formed within the lymphocytes, the membrane fraction was isolated therefrom and found to be similar to the conditioned medium in L929 cytotoxicity and antibody sensitivity (Fig. 7A). As with transfected COS-1 cells, electrophoresis of the membrane material under mild conditions yielded a 90-kDa band reacting with antibodies to Tag7 and to Hsp70 (Fig. 5B).

To ascertain the pathway of the cytotoxic agent, we used Brefeldin A, which is known to irreversibly block protein translocation from endoplasmic reticulum to Golgi (30). Its addition to LAKs during their contact with K562 cells abolished the cytotoxicity of the conditioned medium (Fig. 7A), suggesting that Tag7-Hsp70 is exported through the Golgi machinery.

In parallel, we made use of our finding (under the subsection “ATP Hydrolysis Is Necessary for Tag7-Hsp70 Cytotoxicity”) that metastasin outstripped Tag7 in Hsp70 binding but could not disrupt the assembled Tag7-Hsp70 complex or interfere with its action. Excess Mts1 added to LAKs together with K562 cells (Fig. 7A) or to their membrane fraction prior to solubilization (Fig. 7B) had no effect on cytotoxicity, once again confirming that Tag7-Hsp70 was formed already within the lymphocyte and released in that state into the medium.

DISCUSSION

Recent studies on the peptidoglycan recognition protein family in Drosophila show that PGRP-S is an upstream mediator of the Toll signaling pathway and that PGRP-LC is important for NF-κB activation and immune response (32–35). The functions of Tag7/PGRP-S in mammals have thus far been obscure.

Here, Hsp70 is the first mammalian protein shown to directly interact with Tag7 in solution and in the cell; the fact...
that the two nontoxic proteins form a stable 1:1 complex cytotoxic for several cell lines at very low concentrations strongly supports the specific functional significance of this interaction. The concentration of ATP used to stabilize the Tag7-Hsp70 complex in vitro (5 μM) was chosen to comply with the Hsp70 affinity for ATP ($K_m = 1.3 \mu M$) (36) and to be physiologically reasonable, because effector lymphocytes are known to secrete ATP into the extracellular space in micromolar concentrations (37, 38).

Hsp70 is a classical chaperone but can also act in an ATP-independent way, e.g., it can inhibit c-Jun N-terminal kinase by its peptide-binding domain (14); again, not only full-sized Hsp70 but also the isolated peptide-binding domain and even its crucial tetradecapeptide 450–463 stimulated the cytolytic and proliferative activity of natural killer cells (25). Quite in line with these data, we show that both the large and the small Hsp70 fragments can, albeit far less efficiently, form cytotoxic complexes with Tag7 (Fig. 4B). Considering also that Tag7 does not become cytotoxic by itself when the full-fledged complex dissociates at high ATP (Fig. 4E), we can say that, in this particular case, the role of Hsp70 cannot be reduced to the orthodox chaperoning and driving of Tag7 into the proper conformation. However, full cytotoxicity of Tag7-Hsp70 requires not only the presence of the ATP-binding domain but also the ATPase function (Figs. 4, D and E). The structural details and quantitative aspects of this protein-protein interaction, including the exact role of ATP, are the subjects of further studies.

In our assays, no more than one-third of the target cells died upon exposure to Tag7-Hsp70, regardless of its source, concentration, or the cell line (Figs. 4 and 7). A considerable body of recent data testifies that some genetically homogeneous cell lines derived from tissue cultures are nonetheless substantially heterogeneous in their sensitivity to cytolytic cells (natural killers, cytotoxic lymphocytes) and cytotoxic agents (39–41). It is common knowledge that the TNFα-resistant cell line L929 contains clones resistant to TNFα (39). The resistance of L929r1 was due to the absence of the cell surface receptor for TNFα; in other clones, the intracellular apoptotic signal relay was found to be impaired (42–44). In our hands, limit dilution yielded L929 clones markedly differing in sensitivity to the Tag7-Hsp70 complex (from 8 to 70% cytotoxicity). However, in 7–10 days of culturing the highly susceptible clones reverted to the initial state, which was extremely inconvenient in respect of experimental reproducibility. Similar instability and reversion has earlier been reported for some TNFα-resistant clones (39). Thus, we chose to work with the stable culture in which the overall Tag7-Hsp70 cytotoxicity did not exceed 30%, but the results were well reproducible.

As suggested by the flow sorting data (Fig. 4C), the susceptible cells died by an apoptotic pathway. Hsp70 is conventionally held to be instrumental in cell survival and has more than once been reported to suppress apoptosis (14, 15, 45). To our knowledge, the data presented here for the first time implicate Hsp70 in an apparently opposite process, i.e., cell killing, which, however, may also have a protective function in the organism (such as anti-tumor defense; see below).

The commonly recognized mechanisms of lymphocyte cytotoxicity involve the following: (i) secretion of lymphokines that can exert cytotoxic effects like TNFα (46); (ii) exocytosis of cytolytic granules containing perforin and granzymes (47); and (iii) expression of the Fas ligand (FasL) on the effector cell surface (48). However, this clearly does not exhaust the possibilities of cytotoxic lymphocytes. Thus, hypertrophy and activation of the Golgi apparatus and its migration into the zone of contact with the target cell have been reported (49), although it remained unknown what particular proteins were secreted by the lymphocyte in this case. We have demonstrated previously the calcium-independent secretion of non-granular cytotoxic proteins upon contact with the target (11) and, thereby, returned to exploring the role of the Golgi apparatus in cell killing by cytotoxic lymphocytes. Here we show with transfected (Fig. 5) and natural systems (Fig. 7) that this novel

![Confocal immunofluorescence analysis of BHK21 cells expressing pTag7-EGFP. A and D, cells transfected with pTag7-EGFP. Green indicates localization of fused protein in the cell. E, immunostaining with anti-Hsp70 mouse monoclonal antibodies, followed by Cy3 anti-mouse secondary antibodies. C and F, overlays with yellow indicating co-localization. Both merged images pertain to the Golgi apparatus. Specimens were viewed with a Leica (Wetzlar, Germany) confocal microscope. Images were taken at ×100 magnification.](image)
mechanism involves association of Tag7 and Hsp70 already within the cell, most probably in the Golgi apparatus, and release of the complex through the Golgi machinery (as evidenced by microscopic location (Fig. 6), Brefeldin A blockage (Fig. 7A), and lack of competition from Mts1 (Fig. 7)). We also evidenced by microscopic location (Fig. 6), Brefeldin A blockage (Fig. 7A), and lack of competition from Mts1 (Fig. 7)). We also showed that TNFα is not secreted via Golgi in our system (Fig. 7B). According to our preliminary data, Tag7-Hsp70 secretion is controlled by the Fas-FasL interaction: antibodies to either Fas or FasL suppress the release of the complex. 2 FasL apparently has no transmembrane domain, and it is obscure how the signal secretion is relayed into the cell. We can suppose that, upon contact of the cytotoxic lymphocyte with the target cell, Tag7-Hsp70 is dispatched by Golgi only into the contact zone that arises with the participation of the Fas-FasL interaction. The Fas+ K562 cells, which induce the release of cytotoxic Tag7-Hsp70, are themselves far poorer targets for this complex than the Fas- L292 cells; that is, cytotoxic lymphocytes that kill the Fas+ cells through Fas-FasL interaction concurrently secrete protein complexes capable of lysing the cells devoid of surface Fas. This underscores the universal character of cell killing, but the idea requires more detailed study.

Earlier, the LAK-conditioned medium was found to contain, besides the material corresponding to the complex described here, variable amounts of protein components ranging 55–17 kDa that exhibited cytotoxicity suppressible with antibodies to Tag7 (12) as well as to Hsp70. 2 The most obvious idea is that they originate from the full-sized complex, but we cannot say yet whether this is simply partial degradation or some kind of in vivo processing; work is under way to elucidate the nature and then maybe the function of these entities. Secretion of Tag7-Hsp70 is not unique to CD3+ - and CD8+ -enriched lymphokine-activated killers; for instance, the medium of primary mouse splenocytes exhibits cytotoxicity that can be halved with Tag7 or Hsp70 antibodies. 3

Summing up, the Tag7-Hsp70 complex appears to be an important cytotoxic agent of some lymphoid cells and may represent one of the ways whereby peptidoglycan recognition proteins are involved in innate immunity and anti-cancer defense. Interesting possibilities are suggested, for example, by the overall tumor growth suppression observed upon grafting of autologous tumor cells transfected to produce Tag7 (50).

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