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Aggresome formation by anti-Ras intracellular scFv fragments
The fate of the antigen–antibody complex

Alessio Cardinale, Ilaria Filesi and Silvia Biocca

Department of Neuroscience, University of Rome 'Tor Vergata', Rome, Italy

Diverting the antigen from its normal intracellular location to other compartments in an antibody-mediated way represents a mode of action for intracellular antibodies [Cardinale, A., Lener, M., Messina, S., Cattaneo, A. & Biocca, S. (1998) *FEBS Lett.,* **439**, 197–202; Lener, M., Horn, I.R., Cardinale, A., Messina, S., Nielsen, U.B., Rybak, S.M., Hoogenboom, H.R., Cattaneo, A. & Biocca, S. (2000) *Eur J Biochem.* **267**, 1196–205]. In the case of p21Ras, the sequestration of the antigen in aggregated structures in the cytoplasm of transfected cells leads to the inhibition of its biological function. We have further investigated the intracellular fate of the antigen–antibody complex by analyzing the effect of proteasome inhibitors on the formation and the intracellular localization of the aggregates. Overexpression of anti-Ras scFv fragments or inhibition of proteasomes activity leads to the formation of large perinuclear aggresomes formed of ubiquitinated-scFv fragments in which p21Ras is sequestered and degraded in an antibody-mediated way. Disruption of microtubules by nocodazole completely abrogates the accumulation of scFv fragments in a single aggresome and induces the dispersion of these structures in the periphery of the cell. Cotransfection of the GFP-scFv with a myc-tagged ubiquitin and colocalization with specific anti-proteasome antibodies indicate the recruitment of exogenous ubiquitin and proteasomes to the newly formed aggresomes. Taken together these results suggest that the intracellular antigen–antibody complex is naturally addressed to the ubiquitin–proteasome pathway and that the mechanism of ubiquitination does not inhibit the antibody binding properties and the capacity to block the antigen function.

Keywords: intracellular antibodies; scFv fragment; aggresome; ubiquitin; proteasome.

The intracellular antibody is a strategy to obtain phenotypic knock-out of selected gene products in mammalian cells [1–3]. This technology uses the ectopic expression of recombinant antibodies targeted to different intracellular compartments in order to neutralize intracellular antigens. The function of many antigens has been successfully inhibited by expressing antibodies in the cytoplasm, the nucleus and the secretory pathway of animal and plant cells [4–9]. ScFv fragments expressed from identical expression vectors in the cytoplasm of mammalian fibroblasts have very distinct properties of solubility, stability and tend to aggregate, regardless of the presence of the intracellular antigen [10]. Although some scFvs are soluble cytoplasmic proteins, others are highly concentrated in granular structures whose number, shape and size vary for each scFv and are typical for each antibody.

The phenomenon of aggregation appears to be crucially dependent on the primary sequence of the protein and may be amplified by the very high local concentration of identical nascent molecules which increases the association constants produced by the crowding effect of the accumulation of proteins in the cytoplasm [11–13]. Whether a newly synthetized polypeptide will fold in its native state or aggregate is determined by the competition between the processes of folding, degradation and aggregation. The intracellular pH, the temperature, the redox state, the presence of intracellular chaperones and the inhibition of the degradative pathways are all factors that can greatly influence the aggregation phenomenon and produce folding intermediates prone to aggregation. In a number of reported cases, partially folded or unfolded proteins, which undergo aggregation, accumulate in an intracellular structure, named aggresome [14–16]. This structure, generally formed of ubiquitinated proteins, appears as a pericentriolar membrane-free cytoplasmic inclusion which generates at the MTOC (microtubule-organizing center) and requires intact microtubules to form. The process of aggresome formation is amplified by treatment of the cells with proteasome inhibitors and this suggests that aggregation occurs when the accumulation of unfolded proteins exceeds the capacity of proteasomes to degrade them. Once formed, the aggresomes become very stable, detergent insoluble and refractory to intracellular proteolysis, in spite of the fact that proteasomes are recruited to the site of the aggresome formation.

The aim of our study was to characterize the nature of the aggregating structures formed in scFv transfected cells and to study the fate of the intracellular antigen–antibody complex and its degradation.

To this end we have further exploited the well characterized signal transduction protein p21Ras, point of convergence for different extracellular signal-stimulated pathways [17], which

Correspondence to S. Biocca, Department of Neuroscience, University of Rome, 'Tor Vergata', Via di Tor Vergata 135, 00133 Rome, Italy. Fax: + 39 06 72596407, Tel.: + 39 06 72596428, E-mail: biocca@uniroma2.it

Abbreviations: ALLN, Ac-Leu-Leu-NorLeu; ALLM, Ac-Leu-Leu-Met; scFv, single-chain variable fragment; GFP, green fluorescent protein; NGF, nerve growth factor; CFTR, cystic fibrosis transmembrane conductance regulator; MT, microtubules; MTOC, microtubule-organizing center; FITC, fluorescein isothiocyanate-conjugated; PMSF, phenylmethanesulfonyl fluoride; BrdU, bromodeoxyuridine; PVDF, poly(vinylidene difluoride).

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has been successfully inhibited *in vivo* by the expression of intracellular antibodies [4,5,18–20]. Aggregating anti-p21Ras antibody fragments can sequester the p21Ras antigen by intracellular coaggregation leading to the inhibition of Ras function *in vivo* [18]. Moreover, antigen-specific coaggregation of a number of non-neutralizing scFv fragments with the corresponding protein, has suggested that diverting the antigen from its physiological location by antibody-mediated coaggregation is a general mode of action that could be exploited for intracellular antibody-based phenotypic knock-outs [19]. A further task would be the possibility of addressing the antigen–antibody complexes to the degradative compartment. In this scheme, an antibody targeted to a degradative pathway could be engineered to cause the concomitant transport and degradation of the bound antigen.

Here we describe the effect of proteasome inhibitors on the formation and the intracellular localization of the scFv-formed aggregates. Inhibition of the ubiquitin–proteasome pathway in cells transfected with intracellular scFv fragments leads to the formation of large intracellular aggregates formed by ubiquitinated-scFvs that are reminiscent of the above described aggresomes in several of their morphological and biochemical features. Moreover, exogenously transfected ubiquitin and proteasomes are recruited to these scFv-containing aggresomes, indicating their strict association to the degradative compartment. This process is not dependent on the presence of the antigen, but, in the case of transfection of anti-Ras antibodies, the endogenous p21Ras is sequestered in the aggresomes and degraded in an antibody-mediated way.

Taken together, these results extend the concept of diverting the antigen from its physiological intracellular location by cytosolic antibodies, demonstrating that the antigen–antibody complex is naturally addressed to the degradative compartment of the cell, which, in the case of their overexpression leads to the formation of large aggresomes. Interestingly, the antigen-specific coaggregation and degradation of a number of scFv fragments indicate that the ubiquitination and aggresome formation do not inhibit the antibody binding properties and their capacity to bind and block the antigen function *in vivo*.

MATERIALS AND METHODS

DNA constructs

Constructs pscFvexp-cyt-Y13–259 [anti-(Ras 1)] and pscFv-exp-cyt-α-D11 (anti-NGF) have been cloned as described in Cardinale *et al*. 1998 [18]. The anti-(Ras 2), anti-(Ras 5) and anti-(Ras 6) scFv fragments, derived from Vaughan *et al*. [21] library were subcloned as described previously [19]. All the anti-Ras ScFV fragments equally recognize wild-type and oncogenic p21Ras.

C-myc ubiquitin plasmid pCW7 [22] was a gift from R. Kopito (Stanford University, Stanford, CA, USA).

Y13–259-GFP and α-D11-GFP contructs were obtained by subcloning *Xho*I–*Xba*I fragment from plasmid pEGFPN1 (Clontech) into the pscFvexp-cyt-Y13–259 [anti-(Ras 1)] and pscFvexp-cyt α-D11 (anti-NGF) vectors. Anti-(Ras 2)-GFP and anti-(Ras 5)-GFP were constructed by subcloning the polymerase chain reaction (PCR)-amplified fragment GFP from plasmid pEGFPN1 into the *Not*I–*Xba*I sites of the pscFvex-cyt-anti-(Ras 2) and pscFvex-cyt-anti-(Ras 5) vectors [19].

The following oligos were designed for PCR amplification of the GFP fragment: 5′-ATAAGAATGCGGCCGCGATGGT-GAGCAAGGGCGAGGAG and 3′-GCTCTAGATTACTTG-TACAGCTCGTCCAT. GFP constructs have the green fluorescent protein fused at the C-terminus of the scFv fragments.

All vectors used in this study derive from the pscFvexpress-cyt [23] which direct the expression of cytosolic antibody fragments under the transcriptional control of the EF-BOS (elongation factor-1a) promoter.

Cell lines, transfection and drug treatment

Simian COS and murine 3T3 K-Ras fibroblasts (kindly provided by C. Schneider, CIB, Trieste, Italy) were grown in DMEM medium supplemented with 10% fetal bovine serum.

COS cells were transiently transfected by DEAE-dextran and 3T3 K-Ras were transfected by incubation with Superfect transfection reagent (Qiagen), following the manufacturer's instructions, with a DNA/superfect reagent ratio (w/v) of 1 : 10. Cells were harvested and analyzed 48 h after transfection.

For drug treatments, 25 μM ALLN (Ac-Leu-Leu-NorLeu) and 25 μM ALLM (Ac-Leu-Leu-Met) (Sigma) were added to the culture medium 16 h before harvesting. Lactacystin (Calbiochem) was used at a concentration of 10 μM incubating the cells for 6–7 h before harvesting. Nocodazole was used at a concentration of 1 $\mu g \cdot mL^{-1}$ for 16 h.

Western blot analysis and immunoprecipitation

Transfected cells were washed with NaCl/P_i, lysed for 15 min in ice cold extraction buffer [Tris/Cl 20 mM pH 8, $MgCl_2$ 20 mM, 0.5% NP40, 0,1 $mg \cdot mL^{-1}$ leupeptin, chymostatin and 0.1 mM phenylmethanesulfonyl fluoride (PMSF)] and centrifuged for 15 min (15 000 ***g***). The pellet (insoluble pool) and the supernatant fractions (soluble pool) were analyzed by SDS/PAGE followed by Western blot using the mouse anti-myc IgG 9E10 [24], the monoclonal anti-Ras IgG (pan-Ras Ab-3, Calbiochem) and the monoclonal anti-ubiquitin IgG (Calbiochem) as primary antibodies and horseradish peroxidase-conjugated goat anti-(mouse IgG) Ig (Amersham) as secondary antibodies. The blots were visualized using enhanced chemiluminescence detection kit (ECL, Amersham).

Immunoprecipitation from cellular extracts was performed with mouse anti-myc IgG 9E10 and the monoclonal anti-Ras IgG (pan-Ras Ab-3, Calbiochem) linked to protein A-Sepharose for 2 h at 4 °C. Samples were washed four times in NaCl/P_i/Tris NP-40 0.1% pH 8.0 and once in Tris/Cl 5 mM pH 8.0, boiled for 2 min in SDS-sample buffer and separated by SDS/PAGE. After blotting to a poly(vinylidene difluoride) (PVDF) membrane (Amersham) filters were blocked with 5% milk powder in NaCl/P_i/Tween 0.1% and processed for immunoblotting with the same antibodies used for immunoprecipitation, followed by horseradish peroxidase-conjugated anti-(mouse IgG) Ig (Amersham). Blots were developed using ECL (Amersham).

Immunofluorescence microscopy

Cells were grown on glass coverslips coated with poly L-lysine, then rinsed three times in NaCl/P_i and fixed for 10 min with 4% (w/v) paraformaldeyde (in NaCl/P_i). Cells were permeabilized for 5 min with 0.2% Triton X-100 in 100 mM Tris/HCl (pH 7.5). Incubation with affinity purified mouse anti-myc IgG 9E10, rabbit anti-myc IgG (Boheringer-Mannhein), monoclonal anti-(Ras Ab-3) IgG and rabbit anti-(20S proteasome α subunit) IgG (Calbiochem) was carried out at room

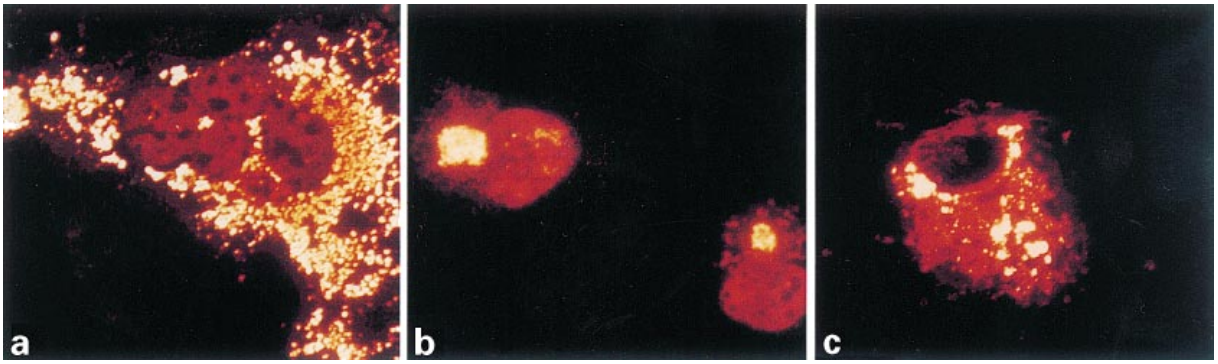

Fig. 1. Effect of proteasome inhibitors on the intracellular localization of scFv fragments. COS cells transfected with scFv α-D11 (anti-NGF) were viewed by immunofluorescence and confocal analysis with anti-myc tag antibodies (monoclonal antibody 9E10). Effect of ALLN: transfected cells were incubated for 16 h without (a) or in the presence of 20 μM ALLN (b). (c) Effect of MT disruption: cells were incubated for 16 h in the presence of 15 μM ALLN together with 1 $\mu g \cdot mL^{-1}$ of the microtubule-depolarizing drug nocodazole.

temperature for 1 h; fluorescein isothiocyanate-conjugated (FITC) goat anti-(mouse IgG) Ig and rhodamine-conjugated goat anti-(rabbit IgG) Ig (Sigma) were used for detection. Samples were routinely examined with a Leitz Dialux 22 microscope, equipped with a 100× oil-immersion lens.

Double immunofluorescence was analysed by confocal microscopy with Leica TCS 4D system, equipped with a 100 × 1.3-O.6 oil immersion objective. Images were recorded with simultaneous excitation and detection of both dyes to ensure the alignment to correct for possible crosstalk resulting from the overlapping of the dyes. Recorded images were corrected, when necessary, with MULTICOLOR analysis package software by Leica and compared with images recorded with single dye excitation. The superimposition of the two chromophores in the same image results in a green/red color scale, that turn yellow in case of colocalization.

Bromodeoxyuridine (BrdU) incorporation

BrdU incorporation of transfected 3T3 K-Ras cells was carried out as described [19] and detected by immunofluorescence incubating the cells with mouse anti-BrdU IgG (Boehringer-Mannhein) containing nucleases for DNA denaturation, followed by anti-(mouse IgG) Texas Red (Calbiochem). BrdU-positive cells were counted from nontransfected and transfected cells. At least 50 positively transfected cells were counted for each cell population in each experiment.

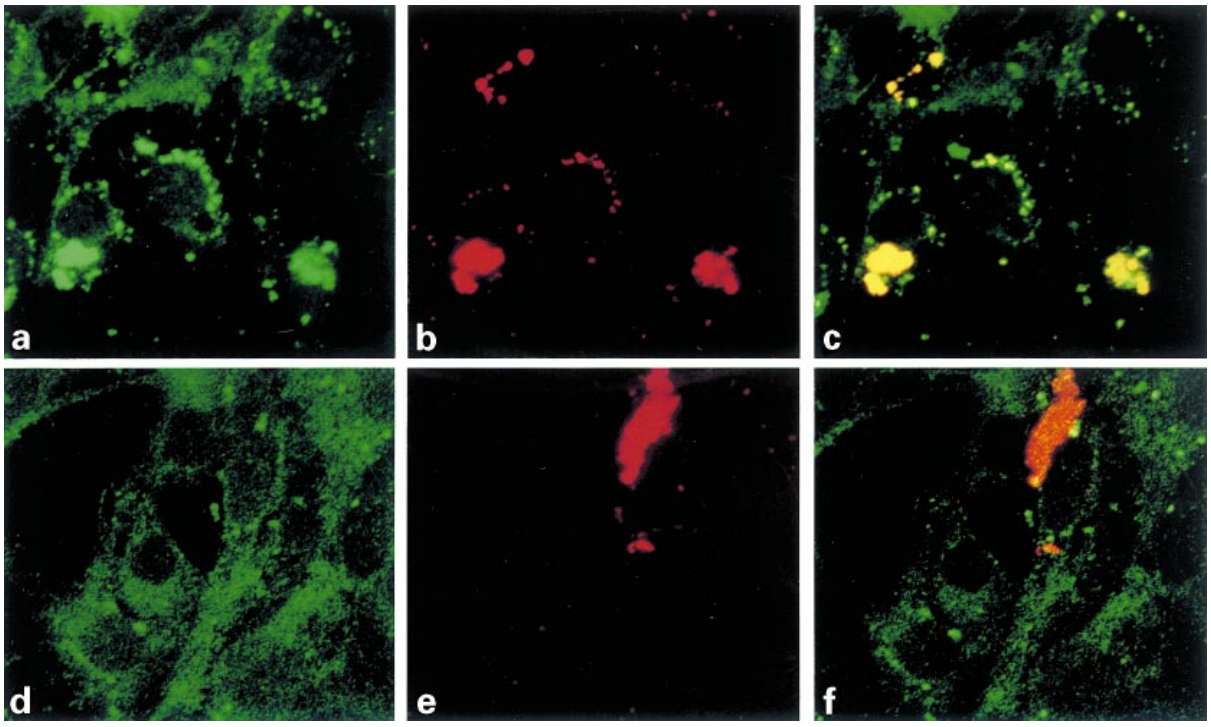

Fig. 2. Colocalization analysis of anti-Ras 5 and anti-NGF scFv fragments with endogenous p21Ras in aggresomes. Confocal analysis of 3T3-K-Ras fibroblasts transfected with scFv anti-Ras 5 (a–c) and scFv anti-NGF (d–f) in the presence of proteasome inhibitor lactacystin (10 μM) for 6 h. Double immunofluorescence with monoclonal anti-(pan-Ras) (a,d) and rabbit anti-myc IgG (b,e) demonstrating the colocalization of the scFv fragment and p21Ras in the aggresomes in the case of cells transfected with anti-Ras 5 (c) and not with anti-NGF (f).

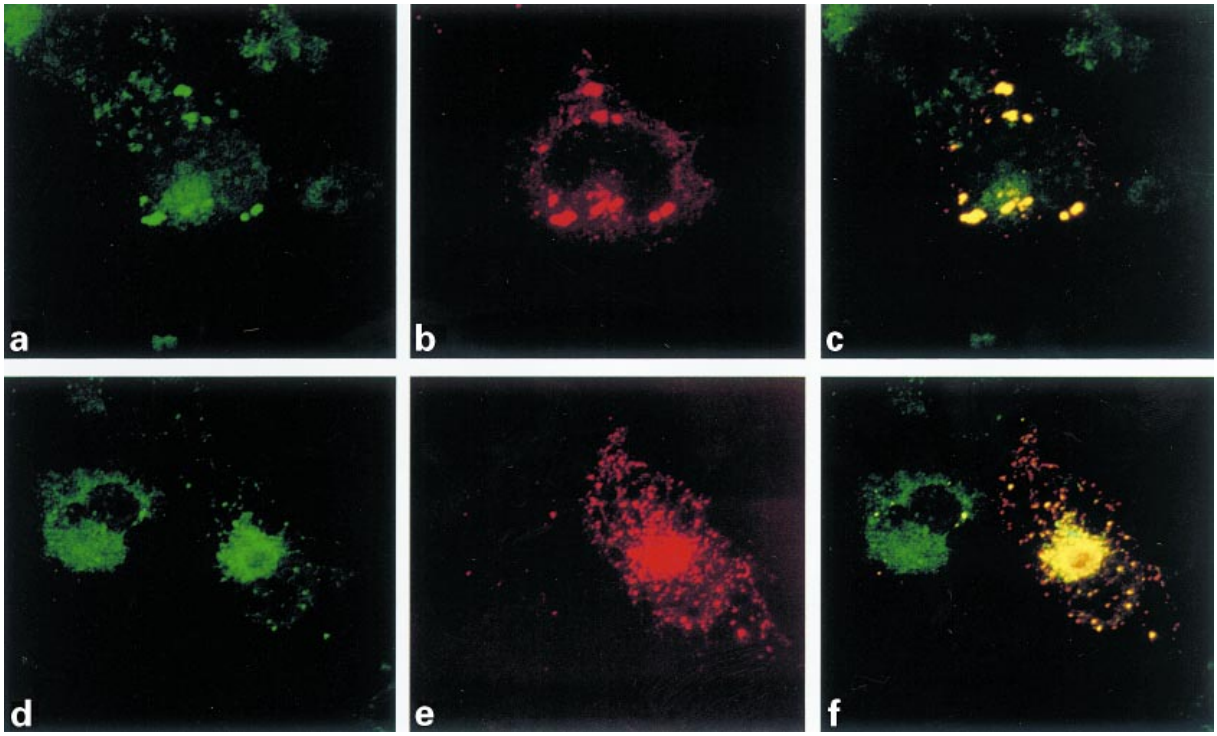

Fig. 3. Colocalization analysis of anti-Ras 5 with the α subunit of 20 S proteasome. Confocal analysis of COS cells transfected with the highly aggregating scFv anti-Ras 5 incubated without (a–c) or with 10 μM lactacystin (d–f). Double immunofluorescence with anti-proteasome IgG (a,d) and anti-myc IgG 9E10 (b,e) demonstrating colocalization of the scFv fragments with the α subunit of the proteasomes in the aggregates (c and f) and in the aggresome (f).

RESULTS

Cytosolic scFv fragments form intracellular aggresomes

In light of the observation that many different scFv fragments tend to aggregate in intracellular structures upon expression in the cytoplasm of mammalian cells, we have investigated the formation and the intracellular location of the aggregates in the presence of proteasome inhibitors. It is well known that the Streptomyces metabolite lactacystin [25,26] and the peptide ALLN (Ac-Leu-Leu-NorLeu, LLnL or calpain inhibitor I) stabilize proteins destined to degradation by the proteasome pathway and lead to the accumulation of ubiquitinated intracellular polypeptides.

COS cells were transfected with scFv fragments, treated with ALLN and analyzed by fluorescence microscopy to monitor their expression. As expected, formation of aggregates was induced also in the absence of proteasome inhibitor (Fig. 1a and [10]). A number of granular or donut-like structures were visible in the cytoplasm, indicating a propensity of this particular scFv fragment to aggregate. Exposure to ALLN led to the coalescence of nearly all these aggregates in a single very large perinuclear structure, as shown in Fig. 1b, which, in many cells, distorted the contour of the nuclear envelope. Another proteasome inhibitor, lactacystin, produced similar results. This large perinuclear structure, which results with the redistribution of the smaller aggregates, is strikingly reminescent to the aggresomes, recently described as ubiquitin-rich cytoplasmic intracellular structures. To further characterize this dynamic aggregation of the small aggregates in a single unit, we treated the transfected cells with the microtubule depolimerizing drug nocodazole, which inhibited the formation of aggresomes leading to the dispersion of several aggregates throughout the cytoplasm [14–16].

As shown in Fig. 1c, treatment of transfected COS cells with the drug nocodazole together with ALLN, completely abrogated the ALLN-induced accumulation of scFv fragments in a single aggresome and induced the dispersion of these structures to the periphery of the cell. Similar results were obtained when other cell lines, as NIH 3T3 fibroblasts, C6 gliomas or Hela cells, were used to express scFv fragments, suggesting that the formation of intracellular aggresomes is not a cell specific response but rather a general mechanism.

These findings suggest that overexpression of cytosolic scFv fragments can trigger the formation of aggresomes.

Diverting p21Ras in the aggresomes using intracellular antibodies

We have recently shown [19] that the expression of a panel of anti-p21Ras intracellular scFv fragments with a different propensity to aggregate in the cell cytoplasm, results in the sequestration of endogenous Ras in the aggregates. In order to verify if treatment with proteasome inhibitors could inhibit the process of diverting and/or sequestration of the antigen, we expressed anti-p21Ras scFv fragments in 3T3-K-Ras transformed cells and induced the formation of intracellular aggresomes in transfected cells. The *in vivo* intracellular interaction between the anti-Ras scFvs and p21Ras was studied by analyzing their intracellular distribution in transfected cells with double immunofluorescence and confocal microscopy.

Figure 2 shows the confocal analysis of 3T3-K-Ras fibroblasts transfected with two highly aggregating fragments, the anti-(Ras 5) scFv (a–c) and the nonrelevant anti-NGF scFv α-D11 (d–f), in the presence of the proteasome inhibitor

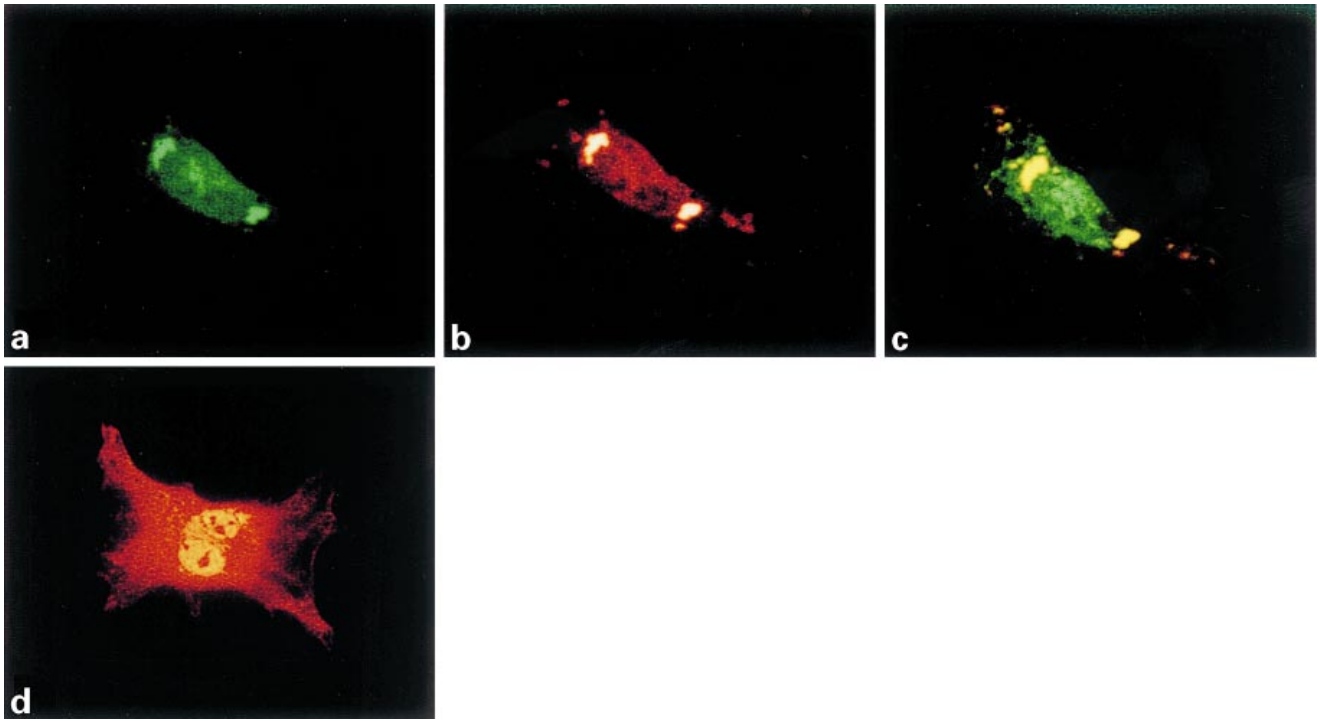

Fig. 4. Colocalization analysis of anti-Ras 5-GFP with myc-tagged ubiquitin. Confocal analysis of COS cells cotransfected with scFv GFP-tagged anti-(Ras 5) and myc-tagged ubiquitin. Autofluorescence of anti-(Ras 5)-GFP is shown in (a), immunofluorescence with anti-myc IgG 9E10 in (b) and merging of the two images in (c). Panel (d) shows cells transfected with myc-tagged ubiquitin alone revealed with anti-myc IgG 9E10 antibodies.

lactacystin. Cells were viewed with anti-(p21Ras) IgG (Fig. 2a,d) and scFv transfected cells were evidentiated with the rabbit anti-myc IgG (Fig. 2b,e). The combination of the two fluorescence patterns is shown in Fig. 2c for anti-Ras and in panel f for anti-NGF expression. In these pictures the two fluorescence signals, green for anti-Ras IgG and red for anti-myc IgG, were combined and the two chromophores were superimposed in the same image with a green/red scale, which turned yellow in the case of colocalization. As evident, Ras is prominently localized in granules in Fig. 2a, but not in Fig. 2d. The yellow colour resulting from the colocalization of endogenous Ras and the anti-Ras scFv fragments is very evident only in those cells transfected with the anti-Ras scFv fragment (Fig. 2c), while p21Ras does not colocalize in the very large aggresome formed by the nonrelevant scFv (red colour in Fig. 2f). As Ras is a cytosolic soluble protein not found in intracellular aggregates in nontransfected or transfected cells with nonrelevant scFv fragments, its translocation in the newly formed aggresomes in anti-Ras transfected cells is antibody-mediated.

The 20S proteasomes are recruited to the scFv containing aggresomes

The proteasome is a large proteolytic complex that plays an important role in a variety of basic cellular processes and is also a component of the quality control machinery that selectively degrades partially folded or misfolded proteins [27–29]. In cells overexpressing CFTR, for example, the proteasomal machinery is recruited to the centrosomal region, the site where the aggresome is forming [15]. In order to determine if scFv fragment-aggresomes also recruit proteasomes, we analyzed the distribution of the α subunit of the 20 S proteasome in cells transfected with a number of different scFvs. in the absence (Fig. 3a–c) and in the presence (Fig. 3d–f) of the proteasome inhibitor lactacystin. Figure 3 shows the double immunofluorescence analyzed by confocal microscopy of transfected COS cells with anti-(Ras 5), a highly aggregating scFv fragment. The scFv transfected cells were viewed with the anti-myc IgG 9E10 (Fig. 3b,e) and the proteasomes were stained with the anti α subunit of the 20 S proteasome (Fig. 3a,d). In normal conditions proteasomes are localized in the cytoplasm and the nucleus of cells (Fig. 3a). Figure 3c,f illustrates the combination of the two chromophores in a single image in which the sites of colocalization are shown in yellow. A strong colocalization between scFv fragments and the α subunit of 20 S proteasomes is clear in both lactacystin treated and nontreated cells. To confirm the colocalization with proteasomes, a number of different scFv fragments specific to Ras or to other antigens were transfected in COS or 3T3 fibroblasts (data not shown).

This result demonstrates that accumulation of anti-Ras scFv fragments in aggregates or aggresome-like structures is accompained by the recruitment of proteasomes to these sites. This finding is consistent with the idea that the aggregates, where the antigen–antibody complexes are found, are likely to represent sites of proteasome digestion.

Cytosolic scFv fragments within the aggresomes are ubiquitinated

The covalent modification by multiple chains of ubiquitin represents the major means for tagging proteins to proteasomal destruction [30]. To also investigate whether the scFv-formed aggresomes are ubiquitin-rich structures we transfected COS cells with anti-(Ras 1), -(Ras 2) and -(Ras 5) scFv fragments together with a plasmid encoding c-myc-ubiquitin (kindly provided by R. Kopito) and observed their intracellular

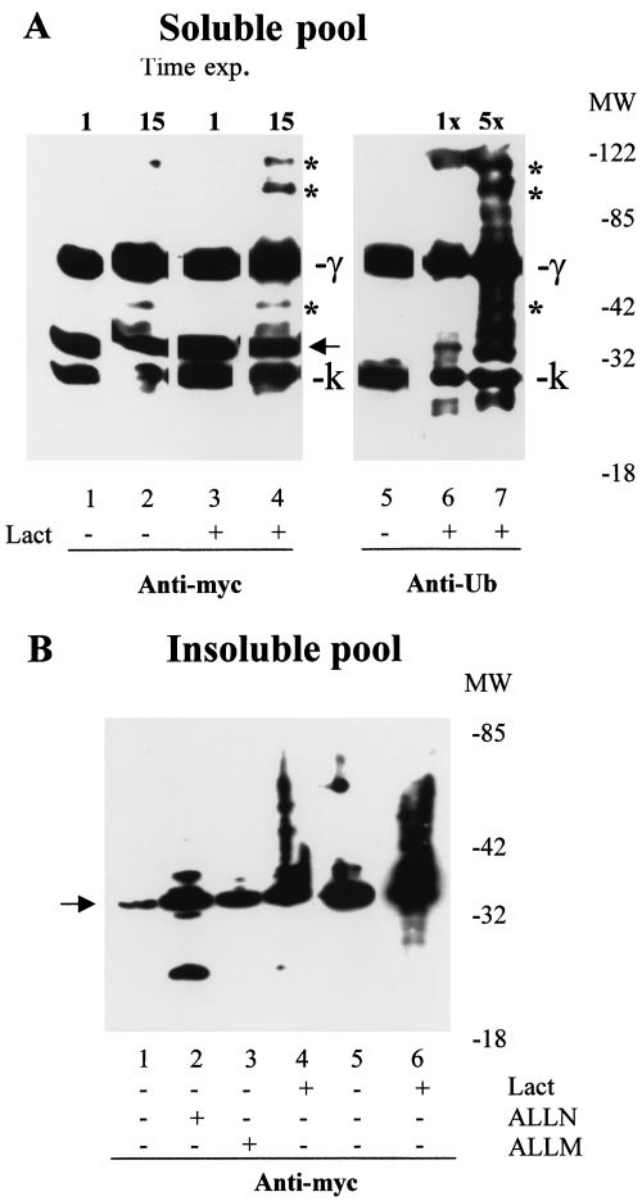

Fig. 5. Identification of ubiquitinated scFv fragments both in soluble and insoluble fractions. (a) Western blot analysis of anti-(Ras 6) scFv fragment immunopurified from transfected COS cells incubated with 10 μM lactacystin (lanes 3, 4, 6 and 7) or not (lanes 1, 2 and 5), viewed with anti-myc IgG (lanes 1–4) and with anti-ubiquitin IgG (lanes 5, 6 and 7). The migration of molecular mass markers (in kDa) and of the heavy (γ) and light (k) chains are indicated on the right. Arrow points to the scFv fragment and the asterisks in lanes 4 and 7 denote three ubiquitinated bands of anti-(Ras 6) scFv fragment. In order to visualize the anti-myc immunoreactive higher molecular mass bands, the ECL plates were exposed for different times (1 and 15 min) as indicated. Overloading the gel up to five times allowed us to highlight the scFv-ubiquitinated bands (lane 7). (b) Western blot analysis with anti-myc IgG of the insoluble pool from COS fibroblasts transfected with anti-Ras 1 (lanes 1–4) and anti-Ras 6 (lanes 5 and 6) incubated with 10 μM lactacystin, 25 μM ALLN and 25 μM ALLM proteasome inhibitors as indicated. Arrow points to the scFv fragments.

localization using a laser scanning confocal microscope (LSCM). Figure 4 shows the colocalization analysis of anti-(Ras 5) and c-myc ubiquitin in COS cells. As it can be seen, the myc-tagged ubiquitin aggresomes (Fig. 4a) clearly turn yellow (Fig. 4c) when superimposed with the cytoplasmic scFv fragment (Fig. 4b). In these cells, the myc-tagged ubiquitin is concentrated in the aggresomes where the scFv fragment is also localized. This result, further confirmed with a number of scFv fragments directed against different antigens, suggests that the soluble scFv fragments are ubiquitinated and degraded, while the insoluble pool remains in an aggregated form. It is important to note that, when the cells are transfected with the myc-tagged ubiquitin alone, the fluorescence is diffuse in the cytoplasm and in the nucleus (Fig. 4d) and that this diffuse fluorescence almost disappears completely in scFv cotransfected cells.

From the colocalization experiments of Figs 3 and 4, it appears that the scFv fragments are specifically cosegregated in aggresomes enriched with ubiquitin and proteasomes. We next studied whether the scFv fragments are themselves covalently linked to ubiquitin. To this end, the soluble and the insoluble fractions of COS transfected cells, treated or not with proteasome inhibitors, were analyzed by Western blot. The immunoreactive bands obtained by blotting the same sample with anti-myc IgG and anti-ubiquitin IgG were compared (Fig. 5) to see if the antibody fragments were ubiquitinated. In this case, we expected to find a ladder of high molecular mass which was immunoreactive to both the anti-myc IgG 9E10 and the anti-ubiquitin IgG.

Soluble proteins were extracted from anti-(Ras 6) COS transfected cells, both treated or not with 10 μM lactacystin for 6 h, and immunoprecipitated with anti-myc IgG 9E10. The cytosolic anti-(Ras 6) scFv fragment, when expressed in mammalian cells, is equally distributed between the soluble and the insoluble fraction. Besides the 32-kDa band corresponding to the the intracellular scFv fragment, at least three higher molecular mass bands are detected by immunoblotting with anti-myc IgG in cells that have been treated with the proteasome specific inhibitor lactacystin (see asterisks in lane 4 of Fig. 5A). A ladder of ubiquitin immunoreactive bands (including the three bands marked with the asterisks) were also visible when the same samples were probed with anti-ubiquitin IgG (lane 7, Fig. 5a). This confirms the presence of ubiquitinated antibody fragments.

Analysis of immunoblot with the anti-myc IgG of the insoluble pool extracted from scFv transfected COS cells is shown in Fig. 5b. Two anti-Ras scFv fragments, the highly aggregating anti-(Ras 1) (lane 1–4) and the anti-(Ras 6) (lane 5 and 6), were used for transfection and cells were treated with 25 μM ALLN and 25 μM ALLM for 12 h and 10 μM lactacystin for 6 h. As it can be seen, the amount of intact scFv fragments increased when transfected cells were treated with protease inhibitors (lane 2, 3, 4 and 6 of Fig. 5b). The proteasome inhibitor lactacystin significantly suppressed the breakdown of the 32-kDa band (lane 4 and 6), leading to the accumulation of the scFv fragment together with a ladder of higher electrophoretic mobility bands. On the contrary, the ALLN proteasome inhibitor, which also inhibits calpain, resulted in the accumulation of both the 32-kDa protein and a degradation product of 24 kDa (lane 2). This result suggests that the scFv fragment is processed by 26S proteasomes and then degraded to smaller peptides by a calpain-like protease. The peptide ALLM only slightly increases the total amount of the intact scFv fragment (lane 3, Fig. 5b) indicating that only a small proportion of the molecule is processed by ALLM-sensitive proteases.

It is worth noting that the immunoreactive ladder of scFv fragments is visible only in cells treated for 6 h with lactacystin (lane 4 and 6) after overexposing the gel at the ECL (15 min)

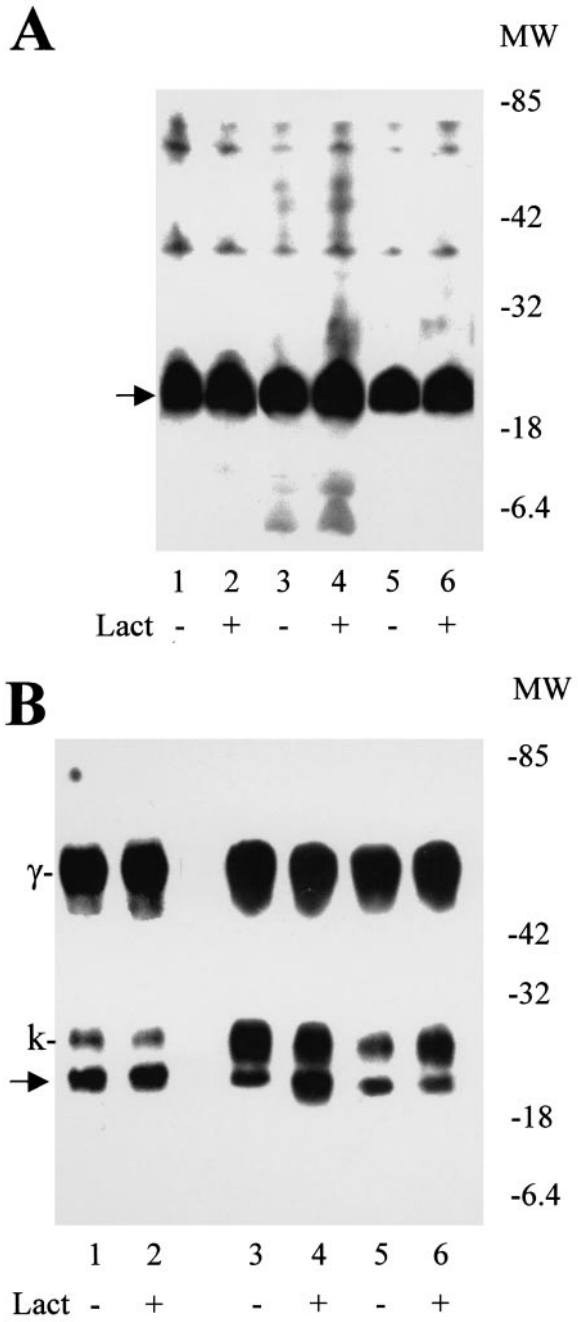

Fig. 6. Identification of p21Ras in fibrolasts transfected with scFv fragments. (a) Western blot analysis of the insoluble fraction of nontransfected 3T3 K/Ras transformed cells (lanes 1 and 2), transfected with anti-Ras 1 (lanes 3 and 4) and with anti-NGF scFv fragments (lanes 5 and 6), incubated with 10 μM lactacystin as indicated and probed with anti-Ras antibodies. (b) Immunoprecipitation of p21Ras from the soluble pool of nontransfected COS cells (lanes 1 and 2), transfected with anti-(Ras 1) (lanes 3 and 4) and with anti-NGF scFv fragments (lanes 5 and 6), incubated with 10 μM lactacystin as indicated and probed with anti-Ras IgG. The migration of molecular mass markers (in kDa) are indicated on the right and the migration of the heavy (γ) and light (k) chains on the left of the gel. The arrow points to the p21Ras protein.

and overloading up to 100 μg of proteins per lane. Neither ALLM (specific to calpains and cathepsins) nor ALLN (inhibitor of proteasomes and calpains) lead to the accumulation of these bands.

P21Ras is addressed to the ubiquitin–proteasome pathway in an antibody-mediated way

The ras gene product is a stable protein with an half-life of about 24 h, depending on the cell line [31] and it is not degraded by the ubiquitin–proteasome pathway in normal conditions. Thus, treatment with lactacystin or ALLN in 3T3 fibroblast or COS cells does not induce the accumulation of endogenous p21Ras.

As, on the contrary, the scFv fragments are ubiquitinated and degraded by the ubiquitin–proteasome pathway (as shown in this study), we addressed the question whether in anti-Ras transfected cells, the antigen p21Ras would follow the same fate. To answer this question, we transfected 3T3 fibroblasts expressing high levels of Ki-Ras and COS cells with anti-(Ras 1) scFv fragment and with the nonrelevant anti-NGF scFv fragments. After 36 h, cells were treated or not for 6 h with 10 μM lactacystin. Cells were then detached from the dishes, washed in NaCl/P_i and counted. Both the detergent-insoluble fractions and the soluble proteins were prepared from the same number of cells, as described in Materials and methods.

p21Ras was analyzed in the insoluble pool of proteins by Western blot and purified by immunoprecipitation with anti-Ras IgG from the soluble pool. p21Ras is a cytoplasmic protein which is partly associated to the inner surface of the plasma membrane and, in the presence of mild detergent conditions is completely recovered in the soluble pool. Only 0.5–1% of the total protein can be recovered in the detergent insoluble fraction of proteins. For this reason, the 3T3 K-Ras transformed cells, highly expressing Ras protein, were used for this experiments and, in order to visualize the degraded p21Ras bands in the insoluble pool, the gels were overloaded and the resulting ECL plates overexposed. The results of these experiments are shown in Fig. 6.

It is worth noting that treatment with lactacystin does not influence the intracellular level of p21Ras in nontransfected cells (Fig. 6a,b, lanes 1 and 2) or in cells transfected with the nonrelevant anti-NGF scFv fragments (Fig. 6a,b, lanes 5 and 6), and confirms the fact that the ubiquitin–proteasome pathway is not involved in Ras degradation. On the contrary, treatment with lactacystin of anti-Ras scFv fragment transfected cells (anti-Ras 1 in this particular case) results in: (a) a significant increase of insoluble (Fig. 6a, lane 4) and soluble (Fig. 6b, lane 4) p21Ras bands, (b) increase of two immunoreactive bands of lower molecular mass in the detergent-insoluble fraction (Fig. 6A, lane 4), and (c) a significant increase in a ladder of higher molecular mass immunoreactive bands in the insoluble pool (Fig. 6a, lane 4). Identical results were obtained by transiently transfecting COS cells with the anti-Ras 5 scFv fragment and with the GFP-tagged anti-Ras 1 (data not shown).

All together, these results suggest that Ras, in the case of anti-Ras transfected cells, becomes sensitive to lactacystin and that this sensitivity is specifically antibody-mediated. The increase of p21Ras in lactacystin treated anti-Ras transfected cells may reflect the increase of the antigen–antibody complexes that are formed and sequestered in these cells. Thus, as previously demonstrated, treatment with lactacystin leads to the stabilization of scFv fragments and induces the formation of scFv-aggresomes. Moreover, the presence of lower molecular mass bands in the insoluble pool, suggests that the sequestered Ras in the scFv-aggresomes is also degraded.

Inhibition of Ras signaling *in vivo* by aggresome formation.

The intracellular antigen–antibody complex tends to aggregate, and this process is greatly increased by the presence of

Table 1. Bromodeoxyuridine incorporation. The table shows the percentage of BrdU positive cells. BrdU positive cells were counted from nontransfected cells and cells transfected with two anti-Ras and with anti-NGF scFv fragments, all of them tagged with GFP. These results represent the average of three different experiments in which at least 50–70 positively transfected cells were counted.

	– Lactacystin (%)	+ Lactacystin (%)
Nontransfected cells	76	75
anti-NGF-GFP	67	65
anti-Ras 5-GFP	27	24
anti-Ras 1-GFP	21	20

protesome inhibitors. To verify if induction of aggresome formation by lactacystin would inhibit or interfere with the Ras-mediated signaling *in vivo*, BrdU incorporation was determined in transiently transfected NIH 3T3-K-Ras. In this assay, we used scFv fragments tagged to the green fluorescent protein (GFP). These chimeras keep their *in vivo* functional activity, giving similar results if compared to the well characterized myc-tagged antibody fragments (A. Cardinale & S. Biocca, unpublished results). It is worth noting that the scFv-moiety in GFP-tagged scFvs was proven to be functional also *in vitro*, as recently reported [32].

Table 1 shows the percentage of BrdU-positive cells expressing different scFv-GFP fragments. At least for the time of incubation used in our experiments, treatment with lactacystin *per se* (10 μM for 6 h) does not interfere with the process of DNA synthesis. Thus, in nontransfected and transfected cells with the nonrelevant anti-NGF scFv-GFP fragment, proteasome inhibition only slightly affects BrdU incorporation. Moreover, although DNA synthesis is inhibited in cells transfected with anti-Ras intracellular scFv-GFP fragments [anti-(Ras 5) and anti-(Ras 1)], this inhibitory activity is not affected by lactacystin.

This finding suggests that the inhibition of Ras function is strictly dependent on its diversion from the plasma membrane to antibody-formed aggresomes and is not augmented by its further degradation in the proteasome-associated aggresomes.

DISCUSSION

The intracellular antibody strategy exploits the ectopic expression of recombinant antibodies to inhibit the *in vivo* function of selected molecules [2]. Folding stability, cellular half-life and the solubility properties of an antibody fragment depend on the intracellular compartment that it is located in. In the case of expression in the cell cytoplasm, the solubility of antibodies is very heterogeneous and formation of intracellular aggregates is a rather frequent event. This may occurr because the reducing environment of this compartment hinders the formation of intrachain disulfide bonds of the variable domains of the heavy and light chains [33]. Our recent demonstration, showing antigen-specific coaggregation of a number of scFv fragments with the corresponding protein, indicates that the intermediate folding state of the cytosolic scFv fragments is native enough to preserve good antigen binding affinity and specificity and that antibody-mediated coaggregation is a general mode of action to obtain antibody-mediated phenotypic knockout [18,19].

The propensity of a protein to aggregate is a natural phenomenon that represents an off-pathway of the normal folding process [34] also occurring during the maturation of newly synthesized proteins. When the concentration of misfolded proteins exceeds the capacity of chaperone and proteolytic systems to degrade them, the aggregates may deposit in the cytoplasm and trigger the formation of large aggresomes as part of a general cellular response to the accumulation of aggregation-prone proteins [14–16,35].

In this study we found that inhibitors of proteasomes in scFv-transfected cells induce the formation of a single perinuclear structure which derives from the coalescence of several scFv-formed aggregates and is highly similar to the aggresome in several biochemical and morphological characteristics. The formation of scFv-aggresomes doesn't depend on the presence of the endogenous antigen but, when the antigen is present, the antigen–antibody complex coaggregates and follows the dynamic redistribution of the newly formed aggresomes in the cytoplasm.

One way to explain the triggering of aggresome formation by scFv fragments is their intrinsic folding properties in the cell cytoplasm [10,33]. As mentioned above, the cytosolic scFv fragments do not form intrachain disulfide bridges and, thus, are found in an unstable molecular state that depends on their intrinsic level of tolerance to correctly assemble even when disulfide bonds don't form. This explains why some scFv fragments are more soluble than others and why, at a threshold level, even the most stable are prone to aggregate. Because aggresomes are a cellular response to the presence of misfolded proteins, and, in particular, seem to form when the degradative pathway is unable to digest all of the misfolded molecules [35], it is plausible that the scFv fragments are recognized by the cells as proteins that must be degraded.

In this paper we show proof that scFv fragments are ubiquitinated and the ubiquitination is seen in both the soluble and insoluble pool. As is well known, the ubiquitination is a sufficient degradation signal to address proteins to the proteasome-mediated degradation pathway [36]. Ubiquitination is not often easy to demonstrate because it is a transient phenomenon and ubiquitinated molecules can be de-ubiquitinated by specific enzymes [37] or quickly degraded by proteasomes. In our specific case, only treatment with proteasomes inhibitors and overexposure of the gel made it possible to evidence the bands of ubiquitinated scFv fragments.

This finding may be in line with a second hypothesis to explain the formation of scFv aggresomes. It is possible that all scFv fragments, notwithstanding their folding properties and/or in light of the fact that they may hinder some criptic signals of degradation, are recognized by the ubiquitin ligase enzymes as substrates and ubiquitinated. The ubiquitinated scFv fragments are rapidly degraded in the cytoplasm but, when their concentration exceeds the capacity of the cells to eliminate them, may become prone to aggregation.

Some findings led us to believe the likelihood of this second hypothesis, even though neither possibility is mutually exclusive. For example, already in the first experiments on the expression of intracellular antibodies in the cytoplasm, we had observed that the half-life of these molecules in the cytoplasm was shorter than in other cell compartments [1,38]. If ubiquitinated soluble scFv fragments are quickly degraded, this would explain why it is not easy to prove the presence of these molecules, especially in experiments which analyze the steady state. Pulse-chase experiments could further develop this interpretation.

An indirect proof of a rapid degradation of ubiquitinated scFv fragments is also provided by cotransfection experiments with exogenous ubiquitin. In cells that coexpress exogenous myc-tagged ubiquitin and scFv-GFP fragments, we observed an

almost complete disappearance of GFP fluorescence signal which suggested that an increase in the concentration of cellular ubiquitin greatly augments degradation of the ubiquitinated scFv fragments (not shown). Moreover, our recent results using anti-Ras scFv fragments equipped with the nuclear localization signal demonstrating that in the nuclear compartment they are more soluble and highly ubiquitinated, strongly suggest that ubiquitination may be a general post-translational modification of scFvs also in the nucleus. Thus, ubiquitination may represent a sort of targeting signal for newly synthetized scFv fragments that will be addressed to the proteasome pathway. The diverting mechanism would be explained when, in the presence of the endogenous antigen, the latter would bind to the ubiquitinated scFv fragment and the antigen–antibody complex be addressed to the degradative compartment.

In the case of p21Ras, shown in this paper, the antigen follows the fate of the antibody in the aggresomes, in spite of the fact that the process of aggregation and formation of aggresomes may occur independently from the presence of such antigen. In fact, the rather stable protein Ras, which isn't degraded by the proteasome, is found in ubiquitin and proteasome rich aggregates only in those cells expressing the relevant anti-Ras scFv fragments.

The activity of Ras as a signal transduction molecule is not only dependent on its structural transition between GDP/GTP bound forms but also requires its association to the inner face of the plasma membrane. Loss of this association, for example by mutations which affect the isoprenylated cysteine in the Ras C-terminus CAAX box, results in loss of Ras activity [39]. Our results support the hypothesis that the intracellularly expressed anti-p21Ras scFv fragments dislodge Ras from its membrane anchorage sites or inhibit its translocation to the membranes and that the dislodged Ras, bound to the intracellular antibody, aggregates and becomes susceptible to proteolytic attack. Proof that Ras dislodged from the membrane can be more unstable and easily degraded has been recently reported [40].

The increase of detergent insoluble Ras and the appearance of lower molecular mass bands in anti-Ras transfected cells, indicate that Ras degradation in aggresomes is an event strictly dependent to the presence of the relevant intracellular antibody. Moreover, these findings may suggest that the aggresomes may retain a residual proteolitic activity and lactacystin is unable to completely inhibit it. This is in line with the recent observation that viral proteins can also be degraded in proteolysis center highly reminiscent to the aggresomes [41].

On the other hand we have also found that proteasome inhibitors increase the level of soluble p21Ras in anti-Ras transfected cells. This may reflect the fact that, in this experimental condition, the scFv fragments are stabilized and, thus, in the case of expression of anti-Ras, this may correspond to an increase of soluble intracellular antigen–antibody complexes. It could be hypothesized that the soluble antigen–antibody complexes which undergo ubiquitination may be degraded as highlighted in scFv aggresomes.

Finally, how relevant is Ras degradation on the capacity of anti-Ras scFv fragments to inhibit Ras function *in vivo*? The finding that lactacystin has no effect on DNA synthesis and on the inhibitory effect of the anti-Ras scFv fragments concurs with the hypothesis that the mechanism of diverting Ras from its cellular location is the event which leads to its inhibition. Our data suggest that subsequent degradation of the already diverted Ras molecules does not augment the inhibitory effect of intracellular antibodies.

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REFERENCES

1. Biocca, S., Neuberger, M.S. & Cattaneo, A. (1990) Expression and targeting of intracellular antibodies in mammalian cells. *EMBO J.* **9**, 101–108.
2. Cattaneo, A. & Biocca, S. (1997). *Intracellular Antibodies: Development and Applications*, pp. 1–196. Springer Verlag, Berlin, Germany.
3. Richardson, J.H. & Marasco, W.A. (1995) Intracellular antibodies: development and therapeutic potential. *Trends Biotechnol.* **13**, 306–310.
4. Biocca, S., Pierandrei-Amaldi, P. & Cattaneo, A. (1993) Intracellular expression of anti-p21ras single-chain Fv fragments inhibits meiotic maturation of *Xenopus oocytes*. *Biochem. Biophis. Res. Commun.* **197**, 422–427.
5. Biocca, S., Pierandrei-Amaldi, P., Campioni, N. & Cattaneo, A. (1994) Intracellular immunization with cytosolic recombinant antibodies. *Bio/Technol.* **12**, 396–399.
6. Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. & Galeffi, P. (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. *Nature* **366**, 469–472.
7. Duan, L., Bagasra, O., Laughlin, M.A., Oakes, J.W. & Pomerantz, R.J. (1994) Potent inhibition of human immunodeficiency virus type 1 replication by an intracellular anti-Rev. single-chain antibody. *Proc. Natl Acad. Sci. USA* **91**, 5075–5079.
8. Mhashilkar, A.M., Bagley, J., Chen, S.J., Szilvay, A.M., Helland, D.G. & Marasco, W.A. (1995) Inhibition of HIV-1 Tat-mediated LTR transactivation and HIV-1 infection by anti-Tat single chain intrabodies. *EMBO J.* **14**, 1542–1551.
9. Marasco, W.A., Haseltine, W.A. & Chen, S.Y. (1993) Design, intracellular expression, and activity of a human anti-human immunodeficiency virus type 1 gp120 single-chain antibody. *Proc. Natl Acad. Sci. USA* **90**, 7889–7893.
10. Cattaneo, A. & Biocca, S. (1999) The selection of intracellular antibodies. *Trends Biotechnol.* **17**, 115–121.
11. Zimmerman, S.B. & Minton, A.P. (1993) Macromolecular crowding: biochemical, biophysical and physiological consequences. *Annu. Rev. Biophys. Biomol. Struct.* **22**, 27–65.
12. Ellis, R.J. & Hartl, F.U. (1996) Protein folding in the cell: competing models of chaperonin function. *FASEB J.* **10**, 20–26.
13. Dobson, C.M. & Ellis, R.J. (1998) Protein folding and misfolding inside and outside the cell. *EMBO J.* **17**, 5251–5254.
14. Johnston, J.A., Ward, C.L. & Kopito, R.R. (1998) Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* **143**, 1883–1898.
15. Wigley, W.C., Fabunmi, R.P., Lee, M.G., Marino, C.R., Muallem, S., De Martino, G.N. & Thomas, P.J. (1999) Dynamic association of proteasomal machinery with the centrosome. *J. Cell Biol.* **145**, 481–490.
16. Garcia-Mata, R., Bebok, Z., Sorscher, E.J. & Sztul, E.S. (1999) Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. *J. Cell Biol.* **146**, 1239–1254.
17. Campbell, S.L., Khosravi-Far, R., Rossman, K.L., Clark, G.J. & Der, C.J. (1998) Increasing complexity of Ras signaling. *Oncogene* **17**, 1395–1413.
18. Cardinale, A., Lener, M., Messina, S., Cattaneo, A. & Biocca, S. (1998) The mode of action of Y13–259 scFv fragment intracellularly expressed in mammalian cells. *FEBS Lett.* **439**, 197–202.

19. Lener, M., Horn, I.R., Cardinale, A., Messina, S., Nielsen, U.B., Rybak, S.M., Hoogenboom, H.R., Cattaneo, A. & Biocca, S. (2000) Diverting a protein from its cellular location by intracellular antibodies. The case of p21Ras. *Eur J. Biochem.* **267**, 1196–1205.
20. Cochet, O., Kenigsberg, M., Delumeau, I., Virone-Oddos, A., Multon, M.C., Fridman, W.H., Schweighoffer, F., Teillaud, J.L. & Tocqué, B. (1998) Intracellular expression of an antibody fragment-neutralizing p21 ras promotes tumor regression. *Cancer Res.* **58**, 1170–1176.
21. Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hoditis, R.A., Wilton, J. & Johnson, K.S. (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat. Biotechnol.* **14**, 309–314.
22. Ward, C.L., Omura, S. & Kopito, R.R. (1995) Degradation of CFTR by ubiquitin-proteasome pathway. *Cell* **83**, 121–227.
23. Persic, L., Righi, M., Roberts, A., Hoogenboom, H.R., Cattaneo, A. & Bradbury, A. (1997) Targeting vectors for intracellular immunisation. *Gene* **187**, 1–8.
24. Evan, G.I., Lewis, G.K., Ramsay, G. & Bishop, J.M. (1985) Isolation of monoclonal antibodies specific for humen c-myc proto-oncogene product. *Mol. Cell Biol.* **5**, 3610–3616.
25. Fenteany, G. & Schreiber, S.L. (1998) Lactacystin, proteasome function and cell fate. *J. Biol. Chem.* **273**, 8545–8548.
26. Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J. & Schreiber, S.L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science.* **268**, 726–731.
27. Voges, D., Zwickl, P. & Baumeister, W. (1999) The 26 proteasome: a molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem.* **68**, 1015–1068.
28. Goldberg, A.L. (1995) Functions of the proteasome: the lysis at the end of the tunnel. *Science* **268**, 522–523.
29. Jentsch, S. & Schlenker, S. (1995) Selective protein degradation: a journey's within the proteasome. *Cell* **82**, 881–884.
30. Ciechanover, A. (1998) The ubiquitin–proteasome pathway: on protein death and cell life. *EMBO J.* **17**, 7151–7160.
31. Ulsh, L.S. & Shih, T.Y. (1984) Metabolic turnover of human c-rasH p21 protein of EJ bladder carcinoma and its normal cellular and viral homologs. *Mol. Cell. Biol.* **4**, 1647–1652.
32. Hink, M.A., Griep, R.A., Borst, J.W., van Hoek, A., Eppink, M.H., Schots, A. & Visser, A.J. (2000) Structural dynamics of green fluorescent protein alone and fused with a single chain Fv protein. *J. Biol. Chem.* **275**, 17556–17560.
33. Biocca, S., Ruberti, F., Tafani, M., Pierandrei-Amaldi, P. & Cattaneo, A. (1995) Redox state of single chain Fv fragments targeted to the endoplasmic reticulum, cytosol and mitochondria. *Bio/Technol.* **13**, 1110–1115.
34. Wetzel, R. (1994) Mutations and off-pathway aggregation of proteins. *Trends Biotechnol.* **12**, 193–198.
35. Kopito, R.R. & Sitia, R. (2000) Aggresomes and Russell bodies: symptoms of cellular indigestion? *EMBO Report* **1**, 225–231.
36. Laney, J.D. & Hochstrasser, M. (1999) Substrate targeting in the ubiquitin system. *Cell* **97**, 427–430.
37. Wilkinson, K.D. (1997) Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *FASEB J.* **11**, 1245–1256.
38. Cohen, P.A., Mani, J.C. & Lane, D.P. (1998) Characterization of a new intrabody directed against the N-terminal region of human p53. *Oncogene* **17**, 2445–2456.
39. Kato, K., Cox, A.D., Hisaka, M.M., Grahm, S.M., Buss, J.E. & Der, C.J. (1992) Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. *Proc. Natl Acad. Sci. USA* **89**, 6403–6407.
40. Haklai, R., Weisz, M.G., Elad, G., Paz, A., Marciano, D., Egozi, Y., Ben-Baruch, G. & Kloog, Y. (1998) Dislodgment and accelerated degradation of Ras. *Biochemistry* **37**, 1306–1314.
41. Antòn, L.C., Schubert, U., Bacik, I., Princiotta, M.F., Wearsch, P.A., Gibbs, J., Day, P.M., Realini, C., Rechsteiner, M.C., Bennink, J.R. & Yewdell, J.W. (1999) Intracellular localization of proteasomal degradation of a viral antigen. *J. Cell. Biol.* **146**, 113–124.