# The mode of action of Y13-259 scFv fragment intracellularly expressed in mammalian cells

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Received 8 October 1998

**Abstract The anti-p21ras Y13-259 single-chain Fv fragment (scFv) neutralizes the activity of p21-ras when intracellularly expressed in different systems. We have studied the mode of action of this inhibition in 3T3 K-ras fibroblasts and demonstrated that (i) this antibody fragment is highly aggregating when cytoplasmically expressed and (ii) the p21-ras antigen is sequestered in these aggregates in an antibody-dependent manner. This co-segregation leads to an efficient inhibition of DNA synthesis. These results suggest that an antigen can be diverted from its normal location inside the cells in an antibody mediated way, prospecting a new mode of action for intracellular antibodies in vivo.**



*Key words:* scFv fragment; Intracellular antibody; Anti-p21ras antibody; Protein solubility

## 1. Introduction

The intracellular antibody technology is based on the idea that antibody chains or domains, if equipped with suitable targeting signals, could be targeted towards new ectopic intracellular sites to neutralize selected gene products [1,2]. This approach has been successfully applied to inhibit the function of several intracellular antigens in the cytoplasm, nucleus and in the secretory pathway of different biological systems [2,3].

One of the problems encountered from the very initial phases of the intracellular antibody work was related to the limited half life of antibodies and of antibody domains expressed in the cell cytoplasm. The reducing environment of the cell cytoplasm hinders the formation of the intrachain disulfide bonds of the VH and VK domains, which are important for the stability of the folded protein. Indeed, the intrachain disulfide bond does not form in scFv fragments expressed in the cytoplasm of eukaryotic cells [4]. The use of vectors optimized for the subcellular targeting of scFv antibody fragments in mammalian cells led to higher expression levels, although this did not always result in higher levels of soluble protein [5]. In this respect, different scFv fragments have distinct properties when expressed in the cytoplasm of mammalian cells, in terms of solubility and propensity to aggregate [6]. While some show a diffuse intracellular distribution, typical of soluble cytoplasmic proteins, others form intracellular aggregates and are concentrated in granular structures, whose number and size vary for each scFv, being typical for each antibody. It is not clear if the aggregating intracellular antibodies are able to interact with the antigen, and, in doing so, they are able to neutralize its function. In view of this fact, there is a great interest in selecting or engineering 'superframeworks' putatively more suitable for intracellular expression [6–8].

The anti-p21ras Y13-259 scFv fragment has been shown to inhibit the insulin induced meiotic maturation in *Xenopus* oocytes [9,10], in a well controlled experimental situation. In this model system both the whole antibody molecules and the scFv fragments are efficiently expressed in the cytoplasm in a soluble form, co-localize with the antigen p21ras in the submembranous compartment, and inhibit its function by neutralization of the epitope (amino acids 60–76) within the switch II region of p21 ras. In this simplest mode of action, soluble intracellular antibodies act as competitive binders, in a stoichiometric ratio with respect to the antigen.

Evidence for the neutralization of p21ras activity in mammalian cells by the intracellular expression of Y13-259 scFvs has also been presented [11,12]. Moreover, as a step towards cancer gene therapy, intratumor transduction of HCT116 colon carcinoma cells with the Y13-259 scFv using an adenoviral vector leads to tumor regression in nude mice [13]. In these studies, neither data on the intracellular interaction between p21ras and the scFv, nor on the solubility state of the intracellular scFv were reported.

In this paper we have expressed the scFv fragment Y13-259 in mouse 3T3 K-ras transformed fibroblasts. We demonstrated that this antibody fragment is highly aggregating in the cytoplasm of mammalian cells, and that, nothwithstanding this fact, the p21-ras antigen is sequestered in these aggregates into an insoluble form. As a consequence, bromodeoxyuridine incorporation assays show that cell proliferation is highly inhibited in Y13-259 transfected cells. These results suggest that the antigen can be segregated from its normal location inside the cells, in an antibody mediated way, prospecting a new mode of action for intracellular antibodies in vivo.

## 2. Materials and methods

### 2.1. Plasmids

For the expression of antibody fragments in mammalian cells, plasmids pscFvexp-cyt-Y13-259 and pscFvexp-cyt-α-D11 were constructed by subcloning the *Nco*I-*Not*I fragments from pcDNAneoY13-259cyt [14,4] and pcDNAneo-α-D11sec [15,4] into the pscFvexpress-cyt vectors [5]. The pscFvexp-cyt-C12 was kindly provided by Ljdia Persic, and directs the expression of the anti-p21-ras scFv fragment C12, isolated from an antibody phage library (Persic et al., manuscript in preparation). All vectors direct the expression of cytosolic antibody fragments under the transcriptional control of the EF-BOS (elongation factor-1a) promoter.

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PII: S0014-5793(98)01369-6

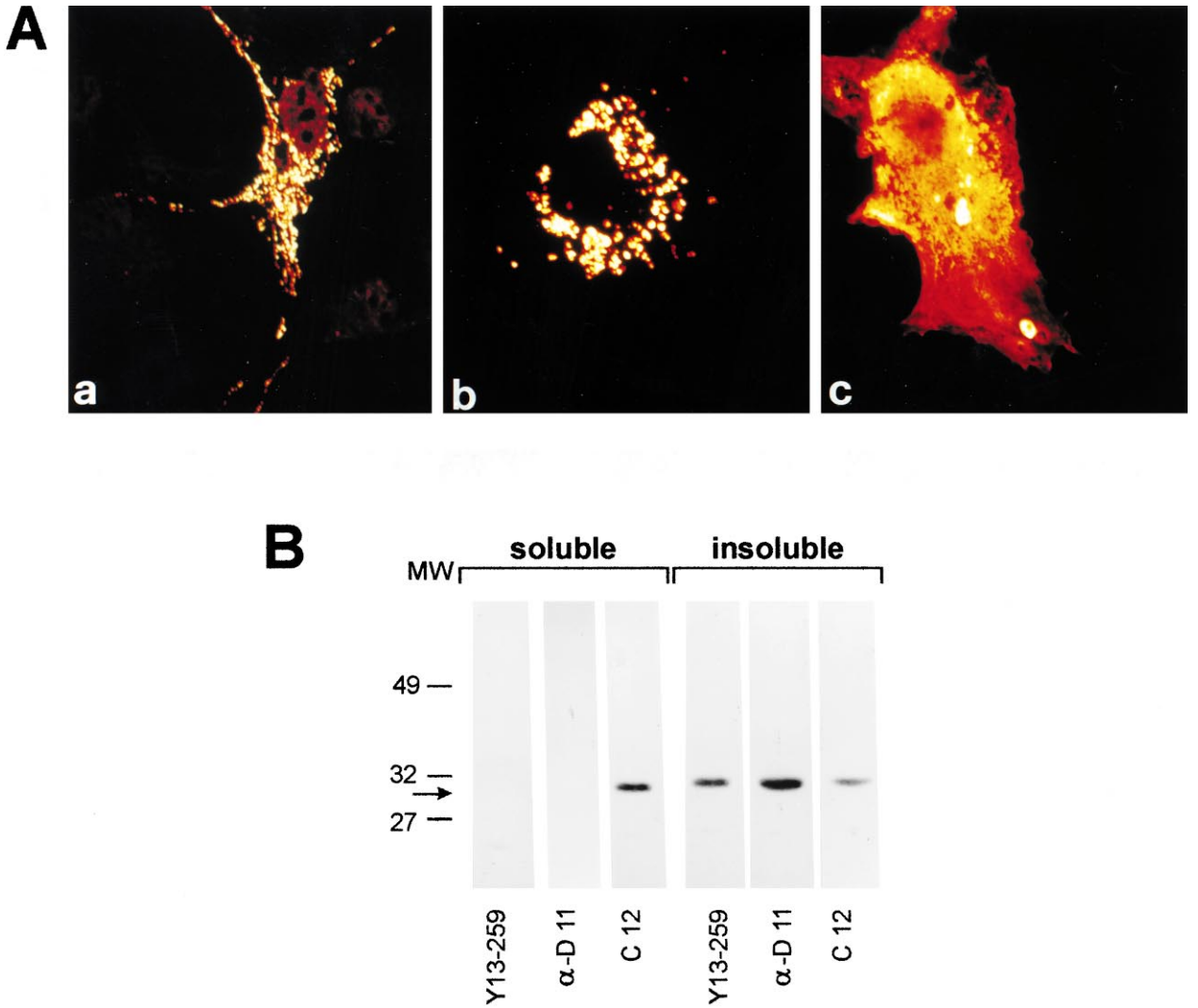


Fig. 1. A: COS cells transfected with Y13-259 (a), α-D11 (b) and C12 (c) scFv fragments targeted to the cytoplasm, viewed by immunofluorescence with anti-myc tag antibodies (monoclonal antibody 9E10) and confocal analysis. B: Western blot analysis of the transfected cell populations shown in A, with anti-myc tag antibodies (monoclonal antibody 9E10). Soluble and insoluble proteins were extracted as described in Section 2. Arrow points to the scFv fragment band.

*2.2. Cell lines, transfection and Western blot analysis*

COS simian and murine 3T3 K-ras fibroblasts (kindly provided by Claudio Schneider) were grown in DMEM medium supplemented with 10% fetal calf 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. Transfected cells were washed with PBS, 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/ml leupeptin, chymostatin and 0.1 mM PMSF) and centrifuged for 15 min (12 000 rpm). The pellet (insoluble pool) and the supernatant fractions (soluble pool) were analyzed by Western blot using the anti-myc mouse monoclonal antibody 9E10 and the human recombinant anti-ras Y13-259 [16] as primary antibodies and goat anti-mouse or goat anti-human horseradish peroxidase (Sigma) as secondary antibody. The blots were visualized by ECL detection kit (Amersham).

*2.3. Immunofluorescence*

Cells were grown on glass coverslips coated with poly-L-lysine, then rinsed three times in PBS and fixed for 10 min with 4% (w/v) paraformaldehyde (in PBS). 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 9E10 and human recombinant anti-ras Y13-259 was carried out at room temperature for 1 h; fluorescein-isothiocyanate-conjugated goat anti-mouse IgG and goat anti-human biotinylated followed by Texas Red streptavidin were used for detection.

Samples were routinely examined with a Leitz Dialux 22 microscope, equipped with a 50× water-immersion objective. Confocal analysis was carried out with a Leica TCS 4D system, equipped with a 100× 1.3–O.6 oil immersion objective.

*2.4. Bromodeoxyuridine (BudR) incorporation*

Transfected 3T3 K-ras cells, after incubation with BudR 10 μM for 24 h, were fixed and permeabilized with 70% ethanol in 50 mM glycine buffer, pH 2, for 30 min at −20°C. BudR incorporation into DNA was detected by immunofluorescence by incubating the cells with mouse monoclonal antibody against BudR (Boehringer-Mannheim) containing nucleases for DNA denaturation, followed by anti-mouse biotinylated antibody (Amersham) and streptavidin-Texas Red (Amersham). BudR positive cells were counted from non-transfected cultures and from pscFvexp-cyt-Y13-259 and pscFvexp-cyt-α-D11 transfected cells. The results shown in Fig. 4 are the average from three different experiments. At least 50–70 positively transfected cells were counted for each transfected cell population in each experiment.

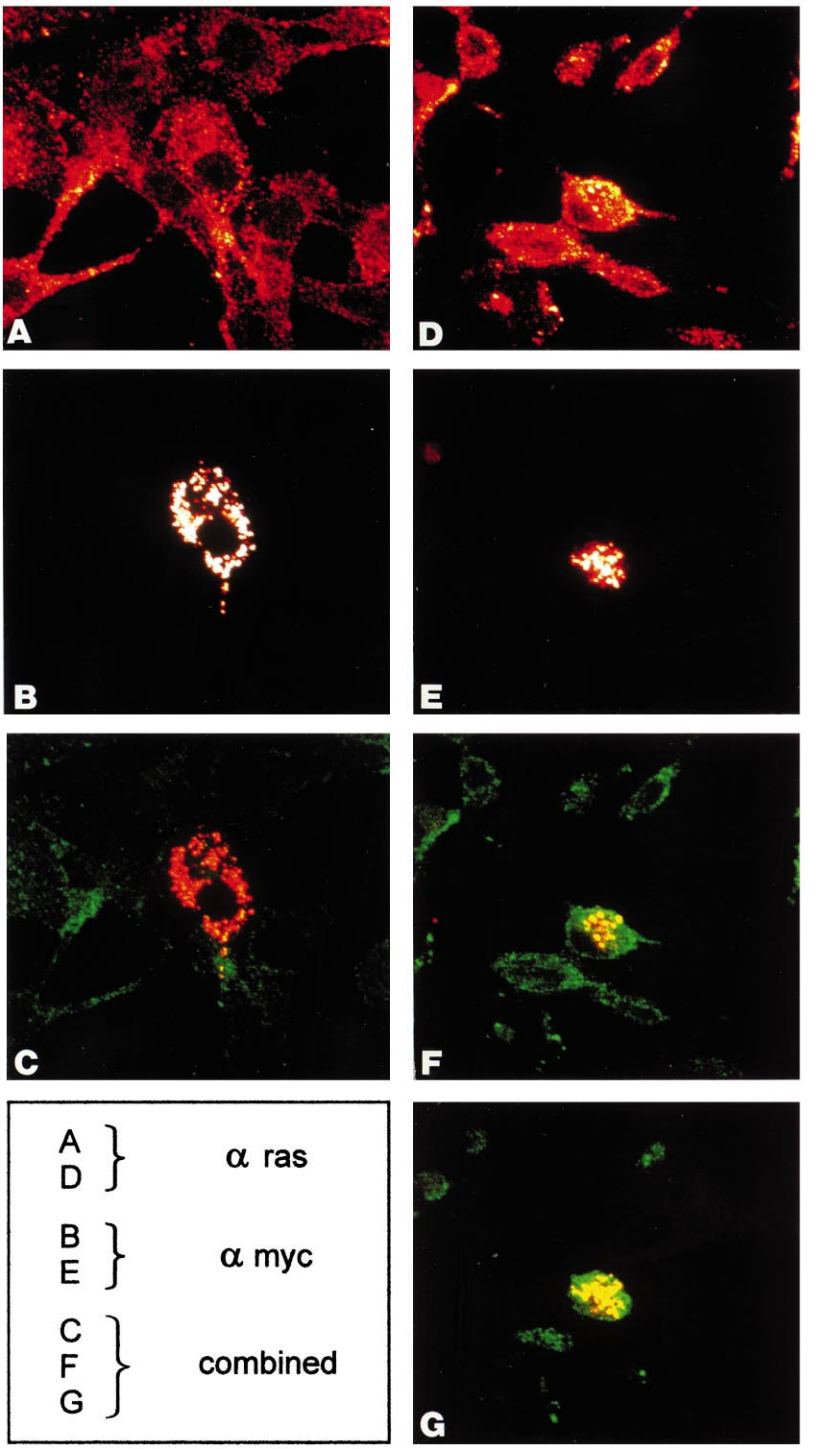


Fig. 2. Double immunofluorescence with anti-p21ras antibody (A,D) and anti-myc (B,E) demonstrating the co-localization of cytoplasmic scFv fragment and p21ras protein only in cells expressing the scFv Y13-259 (F,G), but not in cells expressing the scFv α-D11 (C). A, B and C: 3T3 K-ras fibroblasts transfected with pscFvexp-cyt-α-D11. D, E, F and G: 3T3 K-ras fibroblasts transfected with pscFvexp-cyt-Y13-259. F and G represent two images of a series of the same field.

## 3. Results

Different scFv fragments show distinct propensity to aggregate when expressed in the cytoplasm of mammalian cells. While some show, in immunofluorescence studies, a diffuse intracellular staining typical of soluble cytoplasmic proteins, others are concentrated in granular structures, whose number and size vary for each scFv [6].

### 3.1. *Co-localization of cytoplasmic Y13-259 and p21ras in intracellular aggregates*

In Fig. 1 we have compared the intracellular distribution of

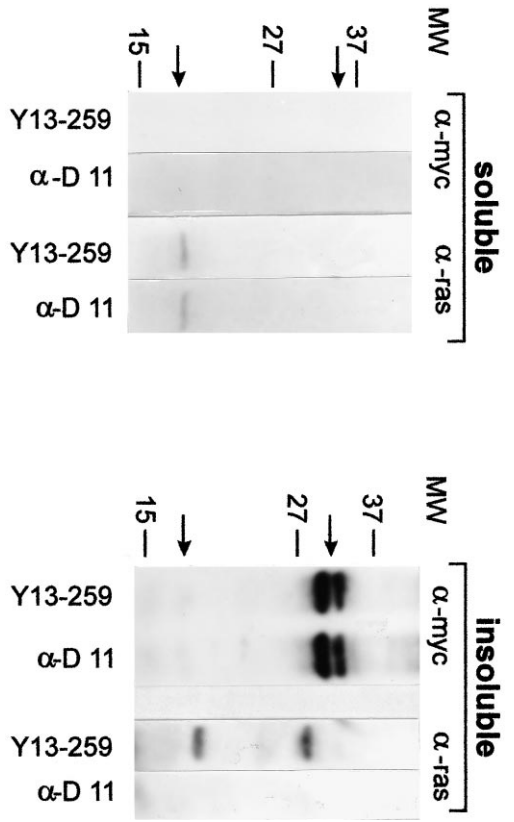


Fig. 3. Western blot analysis of 3T3 K-ras fibroblasts transfected with scFv fragment indicated below each lane and probed with antibodies shown above. Left panel: Soluble pool of proteins. Right panel: Insoluble pool of proteins. The upper arrow indicates the migration of the scFv fragments, while the lower indicates the migration of p21ras protein. Exposure times (ECL detection system) were 1 and 30 min for the left and right panel, respectively. Overexposure of the first two lanes in the left panel up to 100-fold longer times did not reveal the presence of soluble scFv bands.

the anti-p21ras scFv fragment Y13-259 with that of the anti-NGF scFv α-D11, when expressed in COS cells as cytoplasmic proteins. Both scFv fragments concentrate in intracellular granules (Fig. 1A, a and b), a property which is diagnostic of a high insolubility, as confirmed by Western analysis of the soluble and insoluble pool of the two antibody fragments (Fig. 1B, a and b). Under the same cellular conditions, other scFv fragments, cloned in identical vectors harboring the same promoter, show a more diffuse distribution (Fig. 1A, c) and are recovered (mostly) in the soluble pool (Fig. 1B, c), under comparable overall expression levels.

In order to verify whether the intracellularly aggregated Y13-259 scFv fragments are able to interact with the p21ras in the cytoplasm, transformed 3T3 fibroblasts, expressing high levels of Ki-ras, have been transfected with the antibody fragments Y13-259 (anti-p21ras) and with the non-relevant control scFv α-D11. The intracellular distribution of the scFv fragments and of the p21-ras protein was studied in the transfected cells by double immunofluorescence and analyzed by confocal microscopy (Fig. 2A, B and C for α-D11 expression and D, E, F and G for Y13-259 expression). Cells are viewed with anti-p21-ras antibody (Fig. 2A and D) and with anti-myc antibody (Fig. 2B and E). The combination of the two fluorescence patterns is shown in Fig. 2C for α-D11 expression and F and G for Y13-259 expression. In these images the signal viewed with the anti-ras antibody (green) and the signal viewed by anti-myc (red) were combined and the two chromophores are superimposed in the same image with a green/red color scale, leading to yellow color in case of co-localization. As can be seen, in α-D11 transfected cells anti-ras (green) and anti-myc (red) does not co-localize, whereas in Y13-259 transfected cells the two signals appear condensed in the same yellow aggregates.

### *3.2. Segregation of p21ras into an insoluble form*

From the co-localization experiments, it appears that the protein p21ras is specifically segregated in granules in those cells which are positive for the Y13-259 scFv fragments.

To verify whether the scFv fragments and p21ras protein co-localize in an aggregated and insoluble form, the soluble and insoluble fractions were analyzed by Western blot (Fig. 3) of a population of 3T3 K-ras fibroblasts, comprising around 5% of cells transiently transfected with Y13-259 or α-D11. This experiment shows that: (i) both Y13-259 and α-D11 are found in the insoluble pool (Fig. 3, right, lanes 1 and 2) and totally absent in the soluble pool (Fig. 3, left, 1 and 2); (ii) p21ras is a soluble protein and can be detected with anti-ras antibodies among the soluble proteins of both 3T3 cell populations; (iii) interestingly, in extracts derived from 3T3 cells transfected with Y13-259, a consistent fraction of p21ras can be visualized with anti-ras antibodies in the unsoluble pool (Fig. 3, right, lane 3). Pulse and chase experiments with metabolic labeling of cells, followed by immunoprecipitation, confirmed that even the neosynthesized Y13-259 protein cannot be recovered as a soluble protein (data not shown).

This further suggests that the intracellular Y13-259 anti-ras antibodies are interacting in vivo with the p21-ras antigen, diverting it from its normal location and changing its solubility state.

### *3.3. Y13-259 scFv intracellular fragments block DNA synthesis*

p21ras proteins can only exert their stimulation of cellular proliferation when associated to the plasma membrane. Diverting p21ras from its location would result in the inhibition of its function.

To verify whether the expression of the anti-ras scFv Y13-259 would inhibit the signaling for the induction of DNA synthesis, the incorporation of bromodeoxyuridine was determined in 3T3 K-ras cells, transfected with pscFvexp-cyt-Y13-259 and pscFvexp-cyt-α-D11. Fig. 4 shows that the expression of the anti-ras intracellular scFv fragments resulted in a great inhibition of DNA synthesis, with less than 10% of cells entering DNA synthesis, compared to 70–75% of cells non-transfected, or transfected with the anti-NGF antibody fragments. This demonstrates that Y13-259 segregates p21-ras into an insoluble and inactive form.

## 4. Discussion

Although for any given scFv fragment the solubility is partly dependent on the overall expression levels [5], under identical conditions of intracellular expression scFv fragments can have very distinct solubility properties, regardless of the presence of the antigen. Their performance depends primarily on their amino acid sequence, in a way that is as yet unpredictable [6].

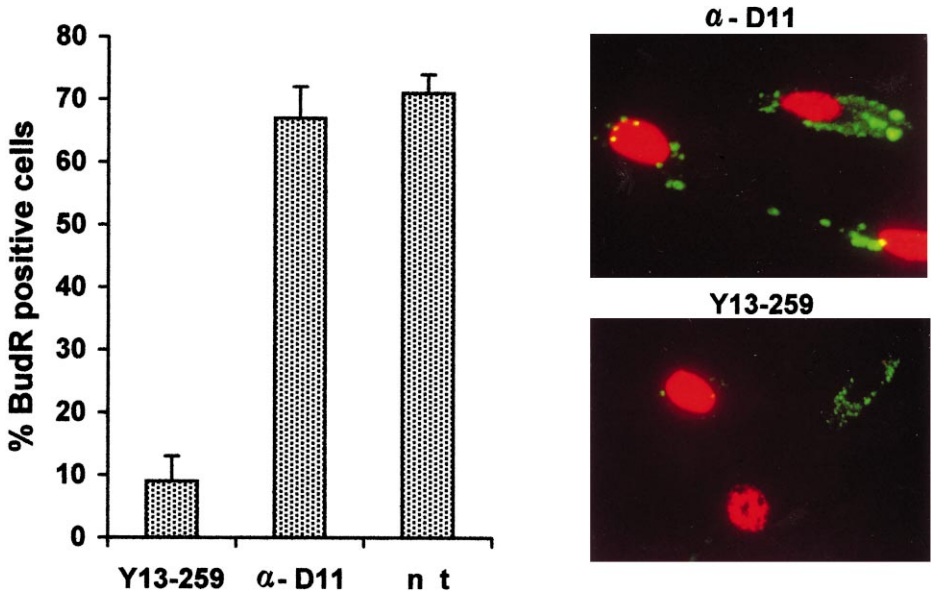


Fig. 4. Bromodeoxyuridine incorporation. BudR positive cells were counted from non-transfected cells (n t), pscFvexp-cyt-α-D11 (α-D11) and pscFvexp-cyt-Y13-259 (Y13-259) transfected cells. The figure shows the average from three different experiments. At least 50–70 positively transfected cells were counted in each experiment.

When expressing proteins ectopically, and scFv fragments in particular, aggregation is often a problem. This is a natural phenomenon that also occurs during the maturation of newly synthesized native proteins and represents an off-pathway of the normal folding process [17]. In some cases the folding intermediate implicated in the aggregation has been shown to have a significant native-like structure, but the generality of this fact remains to be proven. In particular, for aggregating scFv fragments, no binding data on the intermediate folding state are available, also because of the difficulty in acquiring this type of data. This information represents an important issue for the mode of action of intracellular antibodies.

In this paper we have shown that the Y13-259 scFv, as well as other scFv fragments, including the α-D11 anti-NGF scFv, is totally insoluble when expressed in the cytoplasm of mammalian cells. The same scFv was soluble in the cytoplasm of *Xenopus laevis* oocytes [9,10], most likely due to the lower temperature used in those experiments (18°C vs. 37°C). The property of aggregation appears to be shared by different scFv fragments, regardless of the presence of the corresponding antigen. Yet, only cells expressing the aggregated Y13-259 scFv show a dramatic redistribution of the intracellular localization of the p21ras protein, which is now found in the same granular structures as the scFv fragment in a largely insoluble form. It can be concluded that a specific interaction between the co-expressed Y13-259 scFv fragment has occurred, thereby leading to their co-segregation, although the timing of this interaction is still an open question. At steady state, the scFv fragments are shown to be totally insoluble and aggregated, but our results suggest that some intermediate state in the biosynthesis of the cytoplasmic scFv fragment, before it reaches the aggregated state, displays native-like binding properties. This native-like state may be a putative intermediate state representing a bifurcation point in the pathways for folding and aggregation [17]. Following this initial specific binding event, further progress towards aggregation would recruit the p21ras antigen, sequestering it in the aggregate, in a reaction that may be reminiscent of an intracellular immunoprecipitation. It is noteworthy that the aggregated p21ras is detected also using the same Y13-259 antibody, showing that not all the epitopes are engaged with the intracellularly expressed Y13-259. This can be interpreted either by assuming that the 'trapped' p21ras has a multimeric quaternary structure (dimeric or more), or, alternatively, that in the cellular structures where the scFv and the p21ras are selectively co-localized the complex dissociates, possibly due to an unfavorable pH. Attempts to isolate and characterize the cellular granules containing the scFv and the antigen are presently under way.

We have confirmed that the biological action of the p21ras protein is effectively and specifically neutralized in the cells expressing the cytoplasmic scFv, as revealed by inhibition of bromodeoxyuridine incorporation. The intracellular antibodies might act to divert the antigen from its normal location, with a mode of action that leads to a segregation of an antigen in vivo. In this case, the signal for traffic diversion is provided by an aggregating antibody. Learning how to control this process, that may represent an unwanted property of an antibody, will broaden the spectrum of possible modes of action for intracellular antibodies [2]; for instance, learning how the cell disposes or deals with the granules observed under these conditions.

In the case reported in this paper, the scFv fragment is a neutralizing one. It is possible that the neutralization of p21ras occurs, at least partially, prior and independently of the aggregation events leading to the sequestration of p21ras, by a subset of soluble scFv molecules. However, whilst this may certainly contribute to the observed inhibition, it is unlikely to represent the major mechanism of action, given the vast majority of insoluble molecules. Indeed, traffic diversion only needs good and specific binding properties and this mode of action, based on antigen sequestration, eliminates the need of expressing scFv fragments against neutralizing epitopes.

Implementing selection schemes whereby cells are selected on the basis of the phenotype conferred by the intracellular antibodies may help to overcome the potential problems of the aggregating antibodies [18].

The results also have implications for understanding the folding process of antibody fragments under these highly artificial conditions. Whilst it is certainly true that some antibodies fold much better than others under intracellular expression conditions [6–8], the present results prospect the possibility that intermediate folding states may display native-like binding properties. In this case, the intracellular expression has provided a biological read-out for detecting and amplifying a binding event that would not be experimentally accessible in an easy way by other means. The potential for this experimental approach, based on intracellular antibodies, for the study of the folding intermediates is being explored.

*Acknowledgements:* We are grateful to Lyidia Persic for scFv C12 and to Graziano Bonelli for assistance with photographic work. The research described has been supported by an EEC grant (Biotechnology PL 962285), Ministero della Ricerca Scientifica (NE.FAC. within the National Research Plan Neurobiological Systems), Consiglio Nazionale delle Ricerche (CNR) (PF Biotecnologie) and Ministero della Sanità (AIDS Project). M.L. acknowledges EEC for a fellowship.

## References

[1] Biocca, S. and Cattaneo, A. (1995) Trends Cell Biol. 5, 248–252.
[2] Cattaneo, A. and Biocca, S. (1997) Intracellular Antibodies: Development and Applications, Springer Verlag.
[3] Richardson, J.H. and Marasco, W.A. (1995) Trends Biotechnol. 13, 306–310.
[4] Biocca, S., Ruberti, F., Tafani, M., Pierandrei-Amaldi, P. and Cattaneo, A. (1995) Bio/Technology 13, 1110–1115.
[5] Persic, L., Righi, M., Roberts, A., Hoogenboom, H.R., Cattaneo, A. and Bradbury, A. (1997) Gene 187, 1–8.
[6] Cattaneo, A. and Biocca, S. (1998) Trends Biotechnol., in press.
[7] Woern, A. and Plueckthun, A. (1998) FEBS Lett. 427, 357–361.
[8] Proba, K., Worn, A., Honegger, A. and Pluckthun, A. (1998) J. Mol. Biol. 275, 245–253.
[9] Biocca, S., Pierandrei-Amaldi, P. and Cattaneo, A. (1993) Biochem. Biophys. Res. Commun. 197, 422–427.
[10] Biocca, S., Pierandrei-Amaldi, P., Campioni, N. and Cattaneo, A. (1994) Bio/Technology 12, 396–399.
[11] Werge, T.M., Baldari, C.T. and Telford, J.L. (1994) FEBS Lett. 351, 393–396.
[12] Montano, X. and Jimenez, A. (1995) Cell Growth Differ. 6, 597–605.
[13] Cochet, O., Kenigsberg, M., Delumeau, I., Virone-Oddos, A., Multon, M.C., Fridman, W.H., Schweighoffer, F., Teillaud, J.L. and Tocqué, B. (1998) Cancer Res. 58, 1170–1176.
[14] Werge, T.M., Biocca, S. and Cattaneo, A. (1991) FEBS Lett. 274, 193–198.
[15] Ruberti, F., Bradbury, A. and Cattaneo, A. (1993) Cell Mol. Neurobiol. 13, 559–568.
[16] Werge, T.M., Bradbury, A.M., Di Luzio, A. and Cattaneo, A. (1992) Oncogene 7, 1033–1035.
[17] Wetzel, R. (1994) Trends Biotechnol. 12, 193–198.
[18] Gargano, N. and Cattaneo, A. (1997) FEBS Lett. 414, 537–540.