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Intracellular single chain Fv antibody inhibits Ras activity in T-cell antigen receptor stimulated Jurkat cells

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Abstract A mammalian expression vector directing the synthesis of a cytoplasmic single chain Fv version of the Y13–259 anti-Ras antibody was constructed and co-transfected into the human lymphoid cell line Jurkat together with a reporter construct containing the bacterial gene for chloramphenicol acetyl transferase under the transcriptional control of several copies of the binding site for the transcription factor NF-AT. The Ras specific antibody interferes with NF-AT activation upon direct activation of the T-cell antigen receptor, whereas activation by direct protein kinase C stimulation is less sensitive to the anti-Ras antibody. Furthermore, the observed inhibition is dependent on the ratio of antibody to reporter plasmid utilized in the transfection experiments.

Key words: Intracellular immunisation; Ras activity; Jurkat cells; Antibody expression; T-cell antigen receptor

1. Introduction

The involvement of Ras in T-cell antigen receptor (TCR) mediated signal transduction has recently been demonstrated. Activation of the receptor by a specific antigen in association with lymphokine stimulation activate resting T-cells. An indispensable step in this process is the induction of interleukin-2 (IL-2) and interleukin-2 receptor expression, which leads to autocrine growth stimulation [1]. Analysis of the IL-2 promoter revealed that the NF-AT transcription factor is essential for IL-2 expression. It has previously been reported that a synthetic promoter containing several copies of the binding site for NF-AT is activated by TCR stimulation [2].

We have previously demonstrated that activated Ras protein, at least in part, can substitute for direct activation of protein kinase C (PKC) by the phorbol ester, PMA [3]. Furthermore, we have shown that the dominant negative Asn^{17} mutant of Ras, which is deprived of activity and inhibits endogenous Ras [4], abolishes phytohemagglutinin (PHA) activated TCR induced stimulation of NF-AT promoter [3].

Alternative methodologies to interfere with the intracellular function of a biological component have been developed, which involve expression of an antibody intracellularly to allow it to interfere with the biological activity of its antigen. Recently the expression in the secretory compartment of an anti-HIV-1 antibody gene was shown to inhibit correct processing of the HIV-1 gp160 protein, and thus block HIV-1 production [5]. Additionally, this technique has been applied to plants on the expression of anti-artichoke mottled crinkle virus single chain Fv antibody (ScFv) which reduces infection incidence and delays symptom development. [6].

To examine the feasibility of extending this experimental strategy to non-secretory compartments of mammalian cells and to compare its potential to that of a well established dominant negative mutant, we attempted the expression of an anti-Ras antibody molecule in the cytoplasm of Jurkat cells. mRNA microinjection experiments have shown that this antibody protein is correctly folded and interacts with Ras protein in the cytoplasm of *Xenopus* oocytes [7]. Here we show that transfected anti-Ras antibody interferes with TCR induced signal transduction and has an effect comparable to that of the Asn^{17}Ras mutant. We also report that the specificity of the inhibition is similar to that of the dominant negative Ras mutant as also the anti-Ras antibody less efficiently interferes with signal transduction following direct PKC stimulation. Finally we show that blocking Ras signalling by the cytoplasmic anti-Ras ScFv is dependent on the quantities of the ScFv expression vector utilised.

2. Experimental

2.1. Plasmids

The anti-Ras ScFv expression vector, pcDNA1/ScFvY13.259, was constructed as follows. The Y13.259-ScFv gene was subcloned from the secretory bacterial expression vector, p3JF4 [8], as a *Pst*I–*Bam*HI fragment and inserted into the leaderless ScFv expression vector, pJM1. This plasmid has been generated by deleting the pelB secretory leader sequence of p3JF4, thus yielding a leaderless 5′ of the ScFv gene (corresponding to the amino acid sequence Met Gln Val Gln). The leaderless Y13.259-ScFv gene was subsequently cloned into the mammaliam expression vector, pcDNA1amp (Invitrogen) as a *Hin*dIII–*Bam*HI fragment, producing pcDNA1/ScFv-Y13.259.

NF-AT/CAT contains a trimer of the NF-AT binding site upstream of the CAT gene [2]. CMV/gal (Clontech) contains the gene encoding β-galactosidase under the control of the CMV promoter.

2.2. Cell culture, transfections, immunofluorescence and CAT assays

COS-7 cells were maintained in DMEM supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (Gibco). COS cells were transfected with 2μg of the relevant pcDNA1/ScFv vector

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Abbreviations: IL-2 promoter, interleukin-2 promoter; TCR, T-cell antigen receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PHA phytohemagglutinin; CAT, chloramphenicol acetyltransferease; ScFv, single chain Fv antibody; mAb, monoclonal antibody; CMV, cytomegalovirus; Asn^{17}Ras, dominant negative Ras mutant.

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using the Lipofectin Transfection Kit (BRL) according to the manufacturers instructions. After 48 h cells were fixed as described [9] and probed with the mouse anti-myc.tag antibody, 9E10. Specific binding was evidenced using a FITC conjugated anti-mouse Ig antibody (Amity).

Jurkat cells were mantained in RPMI supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (Boehringer, Mannheim). Transfections were carried out as described [3] using 10^6 cells per sample and 0.4 μg NF-AT/CAT and up to 2 μg anti-ras ScFv plasmid DNA or the same amount of control vector per sample. To minimize variability among samples activations were carried out on aliquots of a single pool of transfected cells. In addition all samples were in duplicate. A plasmid encoding β-galactosidase (0.4 μg/sample) was included in all cotransfections as a control of transfection efficiency. Cells were allowed to recover for 22 h, then activated either with 2 μg/ml PHA (Wellcome Diagnostics, Dartford, UK) or with a combination of 10 ng/ml PMA (Sigma) and 100 ng/ml A23187 (Boehringer, Mannheim). Cells were collected 8–10 h after activation and processed for CAT assays as described [10]. Equal amounts of proteins, as determined using the BCA kit from Pierce (Rockford, IL), were used. CAT assays using [^{14}C]chloramphenicol were carried out according to Gorman et al. [10]. Thin layer chromatograms were scanned and chloramphenicol conversion was quantitated using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). CAT values were normalized to β-galactosidase values to correct for variations in transfection efficiency.

3. Results

The Ras specificity of the Y13.259-ScFv was demonstrated after its expression from the vector 3JF4 as a secretory protein in bacteria [8]. However, we considered that the bacterial secretory leader sequence (pelB) in this ScFv vector would not ensure cytoplasmic expression in mammalian cells. To obtain cytoplasmic expression of the ScFv in mammalian cells we deleted the bacterial secretory leader and constructed a leaderless Y13.259-ScFv gene encoding a protein in which the amino terminal is Met Gln Val Gln; Gln corresponds to amino acid number 1 of the heavy chain as defined by Kabat et al. [11]. The leaderless Y13.259-ScFv gene was inserted into the mammalian expression vector pcDNA1amp (Invitrogen) under the transcriptional control of the cytomegalovirus promoter (Fig. 1).

Expression and correct cytoplasmic localisation of the leaderless ScFv protein was assessed in transiently transfected COS-7 cells. The ScFv protein carries the C-terminal myc tag which

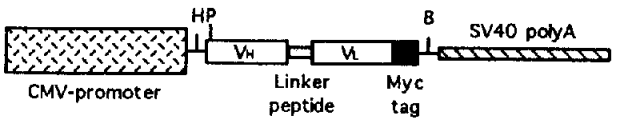

Fig. 1. Mammalian ScFv expression vector. The regions encompassing the CMV promoter, the variable regions (V_H and V_L) and the linker peptide, the myc-tag and the SV40 polyA sequence are shown. Lines indicate non relevant vector sequences. H: *Hin*dIII; P: *Pst*I; B: *Bam*HI.

is specifically recognised by the monoclonal antibody, 9E10 [12]. Indirect immunofluorescence against the Y13.259-ScFv expressed in COS cells yields a staining (Fig. 2A) which is clearly distinct from that of a secretory antibody (Fig. 2B), thus indicating that the protein is correctly expressed outside the secretory compartment. However, unlike the secretory antibody the intracellular ScFv is only seen in a low procentage of the transfected cell population. This phenomenon may reflect specific processing of the immunoglobulin chains and subsequent loss of the myc-tag [13].

To investigate whether that the cytoplasmic anti-Ras ScFv can interfere with Ras dependent signalling we utilised the human lymphoid cell line Jurkat in which T-cell antigen receptor signalling requires Ras activity. A reporter plasmid containing the bacterial gene for chloramphenicol acetyl transferase under the control of several copies of the binding site for the transcription factor NF-AT can be activated in Jurkat cells by treatment with the TCR agonist phytohemagglutinin and induction assessed despite the low number of transfected cells. Cotransfection of the reporter construct with a construct capable of expressing the dominant inhibitor mutant of Ras, Asn^{17}Ras [4], results in inhibition of TCR-induced CAT activity [3]. Fig. 3 shows the results of CAT assays of cells co-transfected with the reporter construct and an expression vector directing the expression of the leaderless anti-Ras ScFv. The levels of CAT activity after PHA stimulation was significantly lower (55%) than in the corresponding control cells (Fig. 3), demonstrating that the Y13.259 ScFv interferes with the TCR activation of NF-AT in a mode similar to that of Asn^{17}Ras. Expression of the Y13.259 also reduced activation of the re-

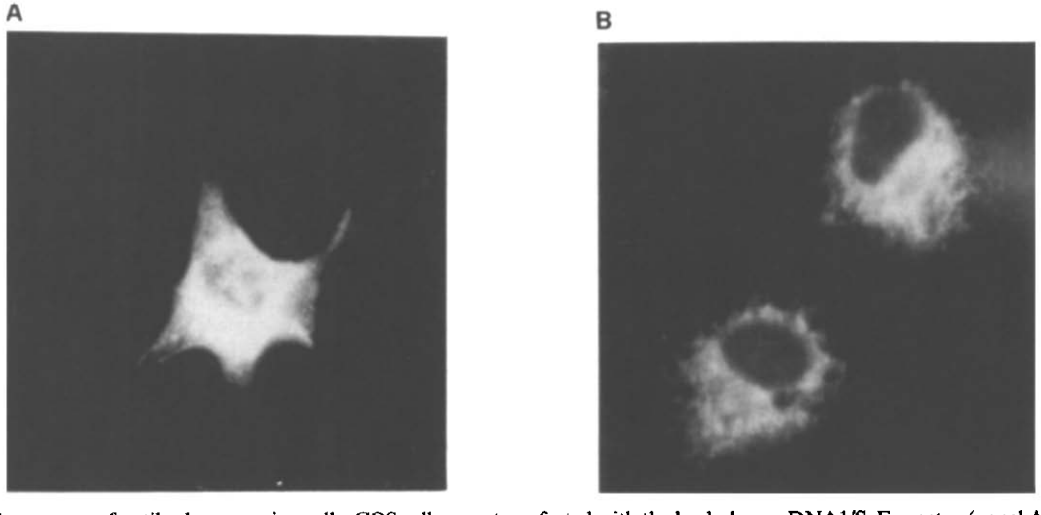

Fig. 2. Immunofluoresence of antibody expressing cells. COS cells were transfected with the leaderless pcDNA1/ScFv vector (panel A) or an antibody with the wildtype Ig secretory leader sequence (panel B). Cells were assayed for intracellular localisation of the expressed antibody protein 48 h after transfection by indirect immunofluresence using the the anti-myc.tag antibody as decribed in section 2.

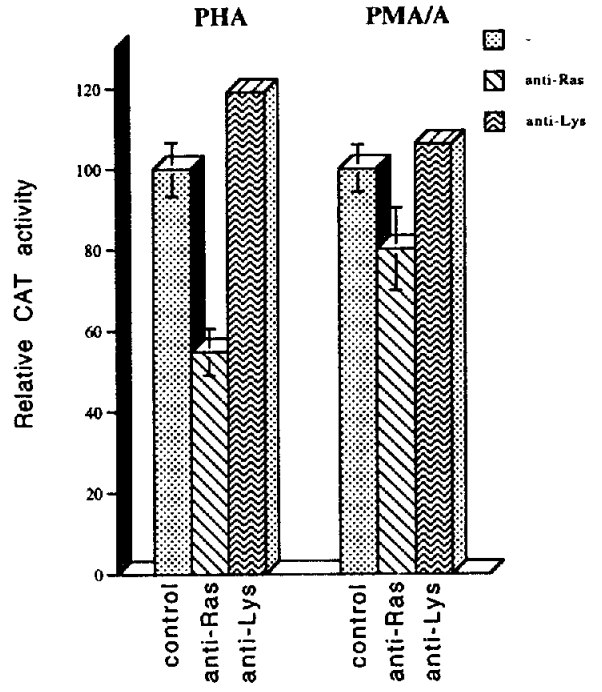

Fig. 3. Inhibition of TCR dependent NF-AT activation by overexpression of anti-ras ScFv. Acetylated [^{14}C]chloramphenicol in thin layer chromatograms of CAT assays of Jurkat cells cotransfected with NF-AT/CAT (0.4 μg/sample) and anti-rasScFv plasmid DNA (1.7 μg/sample) and activated with either PHA or a combination of PMA (10 ng/ml) and A23187 (100 ng/ml) as indicated. Values are expressed as percent of control samples cotransfected with the same amount of empty vector plasmid. Data are average and standard deviation from two independent experiments each with duplicate samples. In one of these experiments, a plasmid containing an irrelevant ScFv (anti-lysozyme) was tested in parallel with the Ras ScFV.

porter by phorbol myristate acetate in combination with the calcium ionophore A231287 but the effect was less dramatic (Fig. 3). Thus, the effect of the anti-Ras ScFv parallels that of Asn^{17}Ras [3] indicating that the Y13.259 ScFv specifically interferes with the activity of the endogenous Ras protein.

Unfortunately, it was not possible to directly assess the levels of the ScFv in the transfected Jurkat cells most probably due to the very low transfection efficiency of these cells and the potential processing of the cytoplasmic ScFv. Nevertheless, the finding that inhibition of PHA stimulated TCR activation of NF-AT is directly dependent on the amount of anti-Ras ScFv expressing vector included in the co-transfection experiment further strengthens the notion that the observed inhibition is Ras-specific. Fig. 4 illustrates that PHA stimulated CAT activity is reduced more than 50% in cells co-transfected with a 5-fold excess of the inhibitory ScFv plasmid with respect to the NF-AT/CAT reporter construct. Further, an inhibitory effect can be detected using as little as 0.5 μg of the anti-Ras ScFv per transfection, representative of a reporter plasmid/ScFv plasmid ratio as low as 1.

4. Discussion

In this study we describe the first application of new method to interfere with a given biological activity in living mammalian cells. We have expressed the single chain Fv derivative of the neutralising anti-Ras antibody Y13–259 as a cytoplasmic protein. The Ras specific ScFv strongly interferes with signal transduction through endogenous Ras protein. Our results demonstrate that the ScFv mediated inhibition parallels that observed with the dominant negative Asn^{17}Ras mutant in that it is considerably more potent in interfering with PHA than PMA/A23187 induced signal transduction. Furthermore, we observe that the inhibition is dependent on the quantity of ScFv DNA utilised in the transfection experiments.

We were unable to completely abolish the TCR signalling through Ras, as it is unlikely that all cells which took up the NF-AT reporter plasmid also took up the ScFv construct. Additionally, it is not known whether at the cellular level there is a linear relation between Ras activity and NF-AT induction, or alternatively that NF-AT activity is only induced in cells where Ras activity exceeds a given threshold. One might speculate that different Ras effects necessitate different levels of Ras activity.

Dominant negative mutations exert their inhibitory action through a competitive interaction with accessory components vital for the activity of the inhibited protein but possibly also for other cellular processes. Therefore, it cannot be excluded that the approach of utilising dominant negative mutations may provide somewhat misleading results. In contrast, the antibody mediated neutralisation results from a direct interaction between the antibody and the Ras protein, thus excluding this possible source of error.

Intracellular immunisation mediated by antibodies expressed in the cytoplasm may provide a very powerful research tool which, contrary to the methologies of anti-sense RNA,

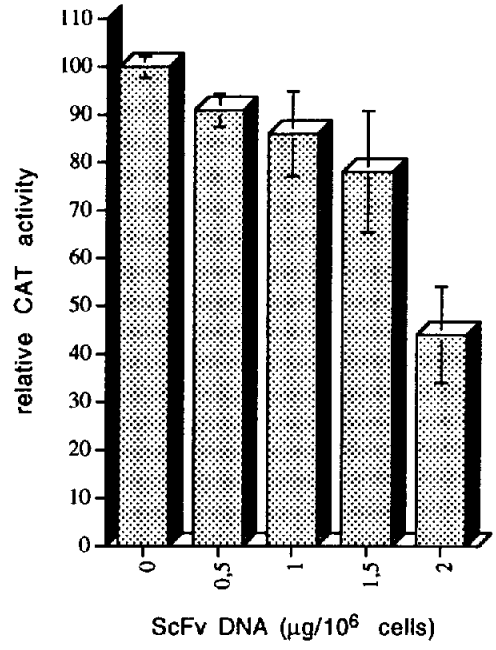

Fig. 4. Dose–response inhibition of TCR induced NF-AT activation by anti-ras ScFv. Acetylated [^{14}C]chloramphenicol in thin layer chromatograms of CAT assays of Jurkat cells cotransfected with NF-AT/CAT (0.4 μg/sample) and increasing amounts of anti-rasScFv plasmid DNA as shown. Control DNA was added to reach a total amount of 2 μg/sample of expression plasmid DNA. Cells were activated with PHA. The figure shows the average and standard deviation calculated on duplicate samples.

ribozymes and dominant negative mutants does not necessitate substantial and individual optimisation, but is based on the expression of one protein from standard vectors. In fact, the general nature of this strategy resides in the characterisation, cloning and expression of one single chain Fv.

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