Immunoregulatory activity of the T-cell receptor α chain demonstrated by retroviral gene transfer

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ABSTRACT We have previously described an antigen-specific I-A<sup>2</sup>-restricted T-cell hybridoma, A1.1, that constitutively releases an antigen-specific immunoregulatory activity into supernatants. Using retrovirally mediated gene transfer, we have found that transfer of the T-cell receptor α chain (TCRα) gene from A1.1 to a number of other T-cell hybridomas effectively transferred the ability to produce the activity. Gene transfer of the TCR β chain (TCRβ), however, did not transfer this ability. The regulatory activity from cells expressing the A1.1 TCRα bound to and was eluted from an anti-TCRα monoclonal antibody and displayed fine antigen specificity identical to that of supernatants from A1.1. The possibility that this activity represents a secreted form of the TCRα (as opposed to shed cell-surface TCR) was examined in BW1100 cells, lacking TCRα and TCRβ, which produced the antigen-specific activity after gene transfer of the A1.1 TCRα gene. The expression of the immunoregulatory activity in supernatants correlated with a direct antigen-binding activity as detected by ELISA, thus raising the possibility that antigen binding is relevant to the mechanism of action of the soluble TCRα. We discuss these observations and our earlier studies suggesting an immunoregulatory role for soluble TCRα.

The heterodimeric T-cell receptor (TCR) has been demonstrated to recognize a ligand composed of an antigenic peptide in association with a major histocompatibility complex molecule (1). Studies in which the TCR α and β chain (TCRα and TCRβ) genes were transferred from one T cell to another have shown that these two peptide chains are both necessary and sufficient for recognition of the ligand (2, 3). Although some studies suggest that the TCR α and TCR β variable regions (V<sub>α</sub> and V<sub>β</sub>, respectively) are skewed toward recognition of antigen and major histocompatibility complex, respectively (4–6), other studies suggest that recognition is an emergent property of the entire receptor (7, 8). It is possible that these divergent views represent differences in the particular TCRs and ligands studied.

Some recent studies (9, 10) suggest that, in addition to its role in T-cell recognition of the antigen and major histocompatibility complex, the TCR might have an immunoregulatory function when shed or released from cells. We have examined this possibility in a number of ways. This laboratory has reported (11) that a helper T-cell hybridoma, A1.1, specific for a synthetic polypeptide antigen, poly-18, plus I-A<sup>2</sup> constitutively releases a poly-18-specific immunoregulatory activity, detected by an in vitro assay (12). The fine antigenic specificity of this cell-free activity corresponded to the specificity of the A1.1 TCR (12, 13). This antigen-specific regulatory activity was bound and eluted from a pan-specific anti-TCRα monoclonal antibody (mAb) and resolved by SDS/PAGE as a 46-kDa protein (13). The activity was not bound by anti-TCRγ, anti-TCR Vα, or anti-CD3ε monoclonal antibodies (13). The relationship to the TCR was further established by the use of antisense oligonucleotides corresponding to TCR Vα that inhibited the production of the soluble regulatory activity (14). Here, we report that retroviral gene transfer of the A1.1 TCRα gene, but not the TCRβ gene, into a number of T-cell lines results in the transfer of the ability to produce the regulatory activity. These results implicate the TCRα as the source of the activity we observe.

MATERIALS AND METHODS

Animals. C57BL/10 and C57BL/6 mice were bred and housed in our facility at the University of Alberta or were purchased from The Jackson Laboratory and housed in our facility at the La Jolla Institute for Allergy and Immunology.

Cells and Reagents. A1.1 (11), B9 (11), BW1100 (15), 175.2 (16), and derivatives of these lines expressing A1.1 TCR genes (see below) were maintained in RPMI 1640 medium plus 10% (vol/vol) fetal calf serum. Cell line 175.2 was the generous gift of Nilabh Shastri (Univ. California, Berkeley). Several of the cell lines were also adapted to a protein-free serum-free medium (Cell Biotechnologies, Rockville, MD). mAbs specific for D3ε (14–2C11 (17)) and the TCR α-chain constant region [H28-710.16 (18)] were purified by protein A affinity chromatography (protein A-Superose, Pharmacia). H28-710.16 was the generous gift of Ralph Kubo (Natl. Jewish Ctr. Immunol. Resp. Med., Denver, CO). Fluorescent staining and flow cytometry analysis of surface CD3 (14) and antibody affinity chromatography with H28-710 (13) were performed as described. Sheep erythrocytes (SRBCs) were purchased from Morose Biologicals (Edmonton, AB) or from Colorado Serum (Denver). The nonrandom synthetic polypeptide poly-18 and peptides based on its structure (listed in Table 1) were the generous gift of Bhagirath Singh (Univ. Alberta, Edmonton, AB).

Retroviral Gene Transfer. All of the retroviral vectors used in this study are derivatives of the N2 vector (19). The A1.1 TCRα and TCRβ cDNAs will be described elsewhere (P. Kilgannon and A.F., unpublished data). In brief, the A1.1 TCRα cDNA uses the Var1.2 (20) and JaTA65 α-chain joining region (20) and the A1.1 TCRβ cDNA uses the Vβ6 (21), Dβ2 β-chain diversity region (22), and Jβ2.7 β-chain joining region (23) gene segments. Expression of the TCRα cDNA was

Abbreviations: TCR, T-cell receptor; TCRα and TCRβ, α and β chains of the TCR, respectively; mAb, monoclonal antibody; SRBC, sheep erythrocyte; Vα and Vβ, α- and β-chain variable regions, respectively; PFC, plaque-forming cell.

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driven by the retroviral long terminal repeat, and the expression of the TCRβ was under the control of the TCR Vβ2 promoter and the TCRβ enhancer (M. V. and A. F., unpublished data). These constructs were transfected into the packaging cell lines ψ2 (24) and PA317 (25) and the titers of the cloned producer cell lines ranged from 5 × 10^5 to 1 × 10^6.

The recipient T-cell hybridomas were infected by producer line supernatants (19) and selected in G418. Further selection was performed in some cases by cell sorting for CD3+ cells using a FACStarPLUS (Becton-Dickinson). The expression of the transduced TCR gene was determined with an anti-Vβ6 (26) or anti-CD3 or by PCR analysis. Primers specific for the Vα1 and α-chain constant region gene segments were used for PCR, and the amplified products were hybridized with an A1.1α junctional region probe.

**Assay for Antigen-Specific Regulatory Activity.** To assay for A1.1-derived antigen-specific regulatory activity, a simple system was employed (12–14). Spleen cells (1 × 10^7 cells) from C57BL/6 or C57BL/10 mice were placed into 1-ml cultures in RPMI 1640 medium with 10% fetal calf serum. Each culture received 50 μl of 1% SRBCs coupled with poly-18 or a substituted polypeptide.Suppressive activity was assessed by adding hybridoma supernatant with or without an “accessory supernatant,” 10–15% (vol/vol), to the cultures. This accessory supernatant was prepared from cultures of murine T cells from animals immunized with SRBCs, followed by absorption of the supernatant with SRBCs, and the supernatant with SRBCs (12, 13). The cultures were incubated at 37°C in humidified 92% air/8% CO2 and anti-SRBC plaque-forming cells (PFC) were assessed 5 days later. In all of the experiments shown, neither the T-cell hybridoma supernatants nor the accessory supernatant significantly affected the immune response when added alone (data not shown). All control and experimental cultures described herein contain accessory supernatant (results without accessory supernatant are not shown).

**Direct Binding of Biotin-Conjugated Peptides to T-Cell-Derived Protein.** The peptides EYK(EYA)EYK and EYKEYAEYAYAAYAEYKY were conjugated to biotin as described (27). Supernatants from cell lines grown in protein-free serum-free medium (see above) were concentrated 50–200 times on a Centricron-30 filtration system (Amicon) and antigen-binding activity was assessed by a modified ELISA assay (28). In some experiments, peptides (without biotin) were added at 100 ng to 1 μg per well with the active biotinylated peptide to assess competition for binding.

**RESULTS**

**Transfer of TCRα from A1.1, With or Without TCRβ from A1.1, Confers the Ability to Produce the Antigen-Specific Activity.** The fine antigenic specificity of the A1.1-derived immunoregulatory activity has been described (12, 13), and those results that are relevant to the experiments described here are summarized in Table 1. As shown in Fig. 1, A1.1 cell supernatants contain an activity capable of inhibiting the anti-SRBC PFC response when SRBCs coupled with EYK(EYA), were present in the culture but not when uncoupled SRBCs or SRBCs coupled with another peptide were present.

Cell line 175.2 expresses TCRβ and the CD3 but lacks a functional TCRα gene (16). The 175.2 cells were infected with a retrovirus expressing the A1.1 TCRα and the cells expressing the A1.1 TCRα (175.2-A1.1α) were selected first in G418 and then by cell sorting of CD3+ cells. The expression of CD3 on the selected cells (Fig. 1A) confirmed that the TCRα was expressed. Supernatants from 175.2-A1.1α cells were collected and tested in the in vitro assay. As shown in Fig. 1B, these supernatants contained the same antigen-specific regulatory activity as the A1.1 supernatant, but supernatant

<table>
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<th>Table 1. Peptides and polypeptides used in this study</th>
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<tr>
<td><strong>Peptide</strong></td>
</tr>
<tr>
<td>[EYK(EYA)4]n</td>
</tr>
<tr>
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</tr>
<tr>
<td>EYKEYAEYAYEYA</td>
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<td>EYKEYAEYAYEYA</td>
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<td>DYTKIMWTPPAIK5S</td>
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Peptides are classified as functional or not functional with A1.1-derived regulatory activity as determined by coupling to SRBCs and testing in the assay system as reported (12, 13). Underscored residues represent substitutions in the basic poly-18 peptide sequence.

from 175.2 cells had no activity. Because the original TCR and specificity of 175.2 are completely unrelated to those of A1.1 (16), these results strongly suggest that expression of the A1.1 TCRα gene results in the production of the antigen-specific regulatory activity.

To further explore this possibility, another cell line, B9, was infected with retroviral vectors carrying the TCRα or TCRβ of A1.1. Like A1.1, B9 expresses both TCRα and TCRβ and produces interleukin 2 in response to the antigen (poly-18) presented with I-A<sup>4</sup> (11). As shown in Fig. 2, supernatants from A1.1, but not B9, contained the antigen-specific regulatory activity. B9 cells expressing the A1.1 TCRα (B9-A1.1α) also produced this activity, whereas those expressing the A1.1 TCRβ (B9-A1.1β) did not. B9 cells expressing both the TCRα and TCRβ from B9-A1.1αβ also produced the regulatory activity.

Supernatants of B9-A1.1α cells were fractionated by antibody affinity chromatography on immobilized anti-TCRα antibody and tested for antigen-specific regulatory activity by using a panel of four peptides coupled to SRBCs in the assay. As shown in Fig. 3, the soluble activity from B9-A1.1α bound to and was eluted from anti-TCRα. The observed specificity for the unsubstituted peptide and the peptide substituted at amino acid 7 (but not those of peptides substituted at residues 3 or 5) is characteristic of the antigen-specific activity from A1.1 (13) (see Table 1). As discussed (13), this specificity correlates with the poly-18 epitope recognized by the A1.1 TCR.

In addition to B9, the A1.1 TCRα gene was transduced into another poly-18-specific cell line, B1.1. After selection with G418, the B1.1-A1.1α lines were found to produce the antigen-specific immunoregulatory activity, although the original cell line (B1.1) does not (data not shown). Thus, two TCRαβ<sup>-</sup> T-cell hybridomas (B9, B1.1) and one TCRαβ<sup>+</sup> T-cell hybridoma (175.2) produced the poly-18-specific regulatory activity after transfer of the A1.1 TCRα gene.

Experiments in which antisense oligonucleotides were used to control TCR expression had previously indicated that the release of the A1.1-derived regulatory activity depends upon expression of TCRα and does not correlate with cell surface expression of CD3–TCR complex (ref. 14 and unpublished observations). To further address whether or not expression of A1.1 TCRα, in the absence of TCRβ, can lead to production of the antigen-specific regulatory activity, we transfected A1.1 TCRα or A1.1 TCRβ into BW1100 cells. Since BW1100 cells lack intact TCRα and TCRβ (15), any effect of TCRα gene transfer should be directly attributable to TCRα. As shown in Fig. 4, supernatants from BW1100-A1.1α, but not BW1100-A1.1β, displayed immunoregulatory
activity. As with the other gene-transfer experiments, this activity showed identical antigenic specificity to that of A1.1.

**Gene Transfer of A1.1 TCRα Correlates with Production of a Direct Antigen Binding Activity.** In earlier experiments on the antigen-specific regulatory activity from A1.1, we found that the active component in the A1.1 supernatant was bound and eluted from an appropriate antigen affinity column (12). If the released TCRα from A1.1 binds directly to antigen, this might be the distinguishing feature that imparts biological activity to this TCRα versus that of other cells. As shown in Fig. 5A and B, supernatants from A1.1 and cell lines expressing A1.1 TCRα contain an antigen-binding component as detected in a modified ELISA assay. This antigen binding was effectively competed by the unlabeled peptide but not by two inappropriate peptides (Fig. 5C), one of which differs from the antigenic peptide by only a single residue. This substitution has been previously shown to abrogate recognition of the peptide by A1.1 (in an antigen-presentation assay) (29) and by the A1.1-derived regulatory activity (13). Thus with our other results, it seems likely that the antigen binding activity is the biologically active product of cells expressing A1.1 TCRα and that it may be the characteristic of antigen binding by this TCRα molecule that imparts its biological activity.

**DISCUSSION**

The CD4+ T-cell hybridoma A1.1 constitutively releases an immunoregulatory activity specific for the synthetic antigen poly-18 and related peptides (12, 13). In this paper, we have demonstrated that gene transfer of the A1.1 TCRα gene into other T-cell lines confers the ability to constitutively produce this antigen-specific regulatory activity (Figs. 1–4). Transfer of the A1.1 TCRβ neither produced nor interfered with this effect (Fig. 2). The antigenic specificity of the soluble activity produced by each transduced recipient T-cell line was iden-

![Image](image.png)

**Fig. 1.** Gene transfer of TCRα from A1.1 to 175.2 cells transfers the ability to produce an antigen-specific regulatory activity. (A) Expression of CD3 on 175.2 and 175.2-A1.1α cells was determined by staining with hamster anti-mouse CD3 followed by fluorescein isothiocyanate-anti-hamster immunoglobulin and examined by flow cytometry. Since 175.2 lacks TCRα (16), expression of cell surface CD3 is indicative of TCRα expression in 175.2-A1.1α. (B and C) Antigen-specific regulatory activity in supernatants of A1.1 and 175.2-A1.1α was detected with SRBCs added to the assay culture coupled to the following peptides. (B) Poly-18 peptide [EYK(EYA)₃EYKY] (solid bars) or control peptides (EYKEYEYAAYAYEYA). After 3 days, the anti-SRBC PFC per culture was determined. Results with uncoupled SRBC (data not shown) were essentially identical to those shown in C.

**Fig. 2.** Gene transfer of TCRα from A1.1 to B9 transfers the ability to produce an antigen-specific regulatory activity. Supernatants from the indicated cell lines were added at a concentration of 10% to assay cultures. SRBCs coupled to poly-18 peptides [EYK(EYA)₃EYKY] (solid bars) or control peptides ([EYA])₃ (open bars) were added to cultures, and anti-SRBC PFC per culture were determined on day 5.

**Peptide coupled to SRBC**

<table>
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<th>Peptide coupled to SRBC</th>
<th>B9-A1.1α Supernatant</th>
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<tbody>
<tr>
<td>None</td>
<td>H28 Filtrate, H28 Eluate</td>
</tr>
<tr>
<td>A1.1</td>
<td>H28 Filtrate, H28 Eluate</td>
</tr>
<tr>
<td>B9</td>
<td>H28 Filtrate, H28 Eluate</td>
</tr>
<tr>
<td>B9-A1.1α</td>
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<tr>
<td>B9-A1.1αβ</td>
<td>H28 Filtrate, H28 Eluate</td>
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**Fig. 3.** Regulatory activity released from B9-A1.1α is bound by anti-TCRα and displays the same fine antigenic specificity as the regulatory activity from A1.1. Supernatants from B9-A1.1α were fractionated by using hamster anti-mouse TCRα mAb (H28) as described (13). Filtrate or eluate was added at 10% (vol/vol) to assay cultures with the indicated peptide coupled to SRBCs. The resulting pattern of fine antigenic specificity is identical to that of the regulatory activity in A1.1 supernatants (12, 13). Underlined residues are substitutions in the poly-18 sequence.
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bound by anti-TCRα mAb, but not anti-TCRβ, anti-TCR Vβ, or anti-CD3ε (13). Further, antisense oligodeoxynucleotides corresponding to different components of the CD3–TCR complex blocked cell surface CD3 expression but not production of the A1.1-derived activity, whereas antisense oligodeoxynucleotides to TCR Vα blocked both (ref. 14 and unpublished observations). Finally, transfer of the A1.1 TCRα gene into BW1100, which lacks TCRβ (15), nevertheless resulted in constitutive production of the antigen-specific regulatory activity (Fig. 4).

Klausner and colleagues (30) have shown that TCRα is retained and degraded in the endoplasmic reticulum unless complexed with CD3ε and further (31) that TCRα that is not exported to the cell surface as part of the CD3–TCR complex is degraded in lysosomes. These observations argue against a pathway whereby TCRα might be released from cells. Recent studies on TCRβ, which is similarly retained and degraded in the endoplasmic reticulum (32), suggest that the assembly and transport of TCR is more complex. For example, in scid/scid mice expressing a TCRβ transgene, TCRβ is clearly expressed on the surface of immature thymocytes in the absence of TCRα or CD3 components (33). Further, a truncated TCRβ gene has been constructed, including only the variable–diversity–joining regions (VDJ) and the β1-chain constant domain, and this is secreted despite the expectation that such a molecule should be retained and/or degraded (34). Thus, the possibility exists that in some cells TCRα might be released in small quantities, possibly in a complex with other unidentified molecules and/or in a posttranslationally truncated form.

We suspect that it is the direct recognition of antigen by the A1.1 TCRα (Fig. 5) that gives this molecule activity in the bioassay and that other T cells might release TCRα that fails to bind directly to the antigen and, therefore, does not display such activity. It is possible, for example, that the complex of TCRα and antigen is immunogenic, resulting in regulatory immune responses to the TCR. Recent studies have indicated that immunization with specific T cells (35, 36) or peptides corresponding to regions in the TCR variable region (37, 38) can result in dramatic immunoregulatory effects in vivo, and it is, therefore, possible that the regulatory effects associated with the A1.1 TCRα might represent a form of such TCR “vaccination” in vitro.

FIG. 4. Expression of the A1.1 TCRα in cells lacking TCRβ is sufficient for production of the antigen-specific regulatory activity. TCRα or TCRβ were transferred to BW1100 cells (15) and supernatants were tested for regulatory activity by using either EYK(EYA)₄EYK (A) or (EYA)₄ (B) coupled to SRBCs.

FIG. 5. Antigen-specific binding activity in supernatants of A1.1 and cell lines expressing A1.1 TCRα. (A and B) Serum-free supernatants of the indicated cell lines were concentrated 50 times and used to coat plastic plates at various dilutions. Plates were washed and incubated with the following biotin-conjugated peptide and developed. (A) EYK(EYA)₄EYK. (B) EYK(EYA)₄EYK. Where the underlined residue indicates substitution in poly-18 sequence. (C) Specificity of the peptide-binding activity in cell supernatants was determined by competitive inhibition. Peptides (see Table 1 for complete sequences) were added at the indicated concentrations to supernatant-coated plastic plates at the same time as the addition of EYK(EYA)₄EYK-biotin and binding of the biotinylated peptide was assessed by A₄₅₀.
Alternatively, it may be that an unidentified molecule associates with the antigen-binding TCRA and this second molecule imparts biological function to the system. For example, Iwata et al. (39) have described a soluble complex of a molecule with glycosylation-inhibitory activity and a molecule bearing TCR determinants released into supernatants of some T-cell hybridomas. Their data are consistent with the possibility that the TCRA imparts antigen specificity and an associated antigen-non-specific molecule determines biological function.

It is interesting that a number of antigen-specific T-cell-derived factors, capable of regulating immune responses in vitro and in vivo, show a relationship to TCRA. Taniguchi and colleagues (40) were the first to demonstrate that TCRA is rearranged and expressed in T-cell hybridomas producing such factors and they found a strong correlation between production of a key lymip hemocyanin-specific factor and expression of a particular Vα2 combination in the T cells (41). Collins et al. (42) have found that T-cell hybridomas that produce factors capable of suppressing immune responses to the hapten nitrophenol express TCRA, and this expression of TCRA, but not that of TCRB, is a requirement for production of the hapten-specific factor. Thus, subclones of a factor-producing T-cell hybridoma that had lost TCRB (and cell surface TCR expression) nevertheless produced the regulatory factor (42), whereas clones that had lost TCRA were unable to produce the factor (V. K. Kuchroo, M. Collins, and M. Dorf, personal communication). Further, they have recently found that transfection of the original TCRA chain into such TCRA mutant restored the ability to produce the factor (V. K. Kuchroo, M. Byrne, M. Collins, and M. Dorf, personal communication). Similarly, a number of laboratories (10, 39, 43) have reported that the antigen-specific component of various T-cell-derived antigen-specific regulatory factors is bound by the anti-TCRA mAb we have used in our studies (ref. 13 and Fig. 3). Since the A1.1 TCRA appears to induce an immunoregulatory activity in vitro, it is possible that other TCRA chains are functional as components of antigen-specific factors with in vivo immunoregulatory activity. If so, this would suggest that our findings may have general implications for the control of immune responses.

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