the voltage sensor, or merely an effect of non-specific partition into membranes is also unlikely because of the parallel effect on charge movement and the h.p.-dependence of the effects.

We have described here effects of nifedipine consistent with an involvement of the high affinity DPRs in E-C coupling. DPRs are generally thought of as Ca channels. It is possible that they are channel-like proteins, not necessarily conducting, that perform the voltage sensing function and are coupled to the SR release pathway by unknown means, involving perhaps a mediator[27].

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Tγ protein is expressed on murine fetal thymocytes as a disulphide-linked heterodimer

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During the search for genes coding for the mouse α and β subunits of the antigen-specific receptor of mouse T cells we encountered a third gene, subsequently designated γ[1]. This gene has many properties in common with the α and β genes[1,2]; somatic assembly from gene segments that resemble the gene segments for immunoglobulin variable (V), joining (J) and constant (C) regions; rearrangement and expression in T cells and not in B cells; low but distinct sequence homology to immunoglobulin *V*, *J* and *C* regions; other sequences that are reminiscent of the transmembrane and intracytoplasmic regions of integral membrane proteins; and a cysteine residue at the position expected for a disulphide bond linking two subunits of a dimeric membrane protein. Despite these similarities the γ gene also shows some interesting unique features. These include a relatively limited repertoire of the germline gene segments[2-5], more pronounced expression at the RNA level in immature T cells such as fetal thymocytes[6-8] and an apparent absence of in-frame RNA in some functional, $\alpha\beta$ heterodimer-bearing T cells or cultured T clones and hybridomas[9,10]. To understand the function of the putative γ protein it is essential to define the cell population that expresses this protein. To this end we produced a fusion protein composed of *Escherichia coli* β-galactosidase and the γ-chain (hereafter referred to a β-gal-γ) using the phage expression vector λgt11 (ref. 11) and raised rabbit antisera against the γ determinants. Using the purified anti-γ antibody we detected a polypeptide chain of relative molecular mass 35,000 (M_r 35K) on the surface of 16-day old fetal thymocytes. The γ-chain is linked by a disulphide bridge to another component of 45K. No such heterodimer was detected on the surface of a cytotoxic T lymphocyte (CTL) clone 2C[12] from which an in-phase γ cDNA clone was originally isolated.

We inserted various parts of the γ cDNA clone pHDS4/203 which contains an in-phase $V_2J_2C_2$ sequence (see ref. 4 and 9 for the nomenclature of mouse γ gene segments) into the *Eco*RI cloning site of the phage expression vector λgt11 and determined the proportion of the β-gal-γ fusion proteins synthesized in the various lysogens by SDS-polyacrylamide gel electrophoresis (PAGE) analysis (data not shown). We then chose the phage construct λgt11-γ1 depicted in Fig. 1*a* because it gave the highest yield of the fusion protein (~4% of the total protein upon induction by isopropyl-thio-β-D-galactoside (IPTG). This γ insert contains almost all the $V_\gamma J_\gamma$ region and part of the *C* region and should code for 15K of the γ polypeptide chain. Figure 1*b* shows Coomassie Blue-stained gels of the lysates prepared from the λgt11-γ1 lysogen without and with induction by IPTG (lanes 2 and 3). The lysate of the λgt11 lysogen with induction is also shown (lane 1). It is apparent from these gel patterns that the 131K band observed in lane 3 is the expected β-gal-γ fusion protein.

The fusion protein was purified by centrifugation followed by preparative SDS-PAGE. As shown in Fig. 1*b* lanes 4-7, the fusion protein can be thus purified close to homogeneity. After removing most of the bound SDS by extensive dialysis, the purified fusion protein mixed with complete Freund's adjuvant was injected subcutaneously into rabbits. Anti-γ-chain antibodies were purified from the sera of hyperimmunized rabbits by first removing anti-β-galactosidase antibody on a β-galactosidase immunoadsorbent and then applying the flow-through to a second immuno-adsorbent composed of the β-gal-γ fusion protein conjugated with Sepharose beads.

To demonstrate that the material eluted from the fusion protein immunoabsorbent indeed contains anti-γ activity we prepared γ protein or fragments of it by two different methods. First, 1 μg of $V_2J_2C_2$ γ RNA was prepared *in vitro* using the SP6 transcription system (Fig. 2*a*)[13,14]. The RNA was then translated *in vitro* into the γ protein using a rabbit reticulocyte lysate. Figure 2*b* lane 1 shows a SDS-PAGE display of the ^{35}S-labelled γ-chain that has been admixed before electrophoresis with the rabbit β globin similarly synthesized *in vitro*. That the purified putative anti-γ antibodies but not the anti β-galactosidase antibody indeed contain anti-γ antibody is demonstrated by the specific immunoprecipitation of the *in vitro* synthesized γ-chain by the former (Fig. 2*b*, lanes 2-5).

Alternatively we prepared a fusion protein composed of mouse immunoglobulin κ chain and the γ-chain constant region by transfecting a myeloma cell J558L with an appropriately constructed chimaeric plasmid. Thus the major C_γ exon and its flanking sequences were inserted in the major intron of a κ light chain gene (Fig. 3*a*). The myeloma cells that have been stably transfected with the plasmid secrete, albeit in small amounts, the $V_\kappa C_\gamma C_\kappa$ three-domain polypeptide chain into the medium

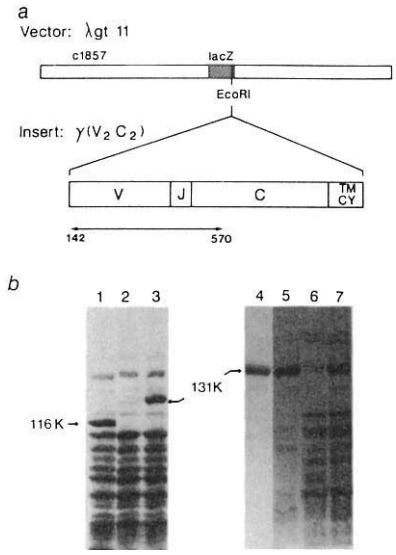

Fig. 1 Synthesis and purification of β-galactosidase-γ fusion protein. *a*, Construction of the chimaeric phage λgt11-γ1. A 429-base pair (bp) DNA fragment coding for the entire *VJ* region and part of the *C* region of the γ cDNA clone pHDS4/203 (ref. 1) was inserted into the *Eco*RI site of the phage λ expression vector λgt11 (ref. 11). *b*, Purification of the fusion protein. Aliquots of the lysates prepared from λgt11 or λgt11γ1 lysogens or fractions thereof were analysed by SDS-PAGE. The gel was stained with Coomassie Blue. Lane 1, λgt11 lysogens induced by IPTG; lane 2, λgt11-γ1 lysogen uninduced; lanes 3 and 7, induced λgt11-γ1 lysogen; lanes 5 and 6, pellet and supernatant fractions (respectively) after a low speed centrifugation of the lysate of the induced λgt11-γ1 lysogens; lane 4, fusion protein eluted from a preparative PAGE run.

Methods. $V_2J_2C_2$ γ cDNA clone pHDS4/203 was digested with *Pvu*II and *Bst*XI and the 429-bp fragment (nucleotide positions 142–570; see ref. 3 for the numbering system used) was purified, blunt-ended by T_4 polymerase, and ligated into the unique *Eco*RI site of λgt11 DNA using *Eco*RI linkers. The recombinant DNA was packaged *in vitro* using the λ phage packaging mix (Promega) and the packaged phages were plated on *E. coli* Y1088 (sup F^+). The phages containing the γ DNA sequence in the appropriate orientation were identified by plaque hybridization followed by restriction enzyme analysis of the purified phage DNA. The lysogens prepared with these phages as well as the vector phage λgt11 were grown and the β-galactosidase or the β-gal-γ fusion gene was induced with IPTG by the standard procedure[11]. The bacterial cells were collected in a lysis buffer (0.2 M Tris–HCl *p*H 7.6, 0.2 M NaCl, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol (DTT), 1 mM PMSF (phenylmethylsulphonyl fluoride) and 100 units ml^{-1} Aprotinin and disrupted by freezing and thawing, then sonication. The lysate was centrifuged at 20,000*g* for 40 min at 4 °C. The pellet was washed once with the above buffer. For the preparative SDS-PAGE the washed pellet was suspended in the lysis buffer. The proteins were dissolved by addition of one volume of 2-fold concentrated SDS-PAGE sample buffer (160 mM Tris-HCl *p*H 6.8, 4% SDS, 20% glycerol, 4 mM EDTA, 10% β-mercaptoethanol) and electrophoresed in 6% cylindrical acrylamide gels of 15 mm diameter. The 131K protein was electroeluted in the electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and dialysed against a buffer containing 50 mM Tris-HCl *p*H 7.5, 2.5 mM octylglucoside, 0.1 mM PMSF, followed by the same buffer without octylglucoside.

along with the endogenous λ light chain (Fig. 3*b*, lane 1). The results shown in the rest of Fig. 3b show that the fusion protein is specifically precipitated with the serum of the rabbit hyperimmunized with the β-gal-γ fusion protein. These results not only confirm the rabbit serum contains anti-γ-antibody but also demonstrate that at least some of this antibody is directed against the C_γ region.

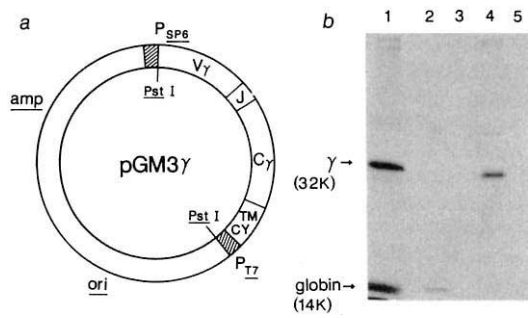

Fig. 2 Immunoprecipitation of the *in vitro*-synthesized γ polypeptide chain by the putative anti-γ antibody. *a*, Construction of a plasmic, pGM3γ, for the *in vitro* transcription by SP6 RNA polymerase. The γ cDNA pHDS4/203 was subjected to limited digestion with *Pst*I and the 1372-bp fragment (nucleotide positions 43-1414 (ref. 3)) containing the entire translated region and some flanking untranslated regions of the γ cDNA was ligated to the *Pst*I site of pGEM3 (Promega). This vector carried the transcription promotor for SP6 RNA polymerase just upstream of the *Pst*I site. *b*, A SDS-PAGE autoradiogram of the immunoprecipitate of the *in vitro*-synthesized γ polypeptide chain. The ^{35}S-labelled γ protein synthesized *in vitro* was mixed with the similarly synthesized rabbit β globin (lane 1) and precipitated with a rat anti-rabbit-globin (lane 2), a normal rat serum (lane 3), the anti-γ fraction (lane 4) and anti-β-galactosidase fraction (lane 5) of the serum of the rabbit hyperimmunized with the β-gal-γ fusion protein.

Methods. pGM38 DNA was linearized with *Bam*HI transcribed by SP6 RNA polymerase (Promega) as described[13,14]. The template DNA was digested with RNase-free DNase and the RNA was extracted by a phenol/chloroform mix (1:1), precipitated by 2.5 volumes of ethanol, and extensively washed with 80% ethanol. The purified γ RNA was translated into ^{35}S-labelled protein using the lysate of rabbit reticulocytes (Promega) according to the procedures recommended by the supplier. In order to raise the anti-β-gal-γ fusion protein, rabbits were immunized subcutaneously with the 1 mg purified fusion protein with complete Freund's adjuvant. After three weeks the rabbits were boosted with 1 mg of the fusion protein with incomplete Freund's adjuvant and bled 7 d later. The immunoglobulin was precipitated from the antisera with one volume of saturated ammonium sulphate, dialysed extensively against a phosphate-buffered saline (PBS) and passed through a column composed of β-galactosidase (Sigma)-conjugated Sepharose 4B. The material adsorbed was eluted from the column with 0.2 M glycine-HCl buffer (*p*H 2.6) (anti-β-galactosidase fraction). The effluent was passed through a second affinity column containing the purified fusion protein and the material adsorbed (anti-γ fraction) was similarly eluted. Immunoprecipitation was carried out by standard procedures using protein A-Sepharose.

We next investigated whether a bona fide γ protein could be detected on murine cells of the T-lineage. We chose fetal thymocytes and a CTL clone 2C because these cells were previously shown to contain a relatively high level of γ RNA[1,6–8]. Furthermore the 2C γ RNA was shown to be in-phase[1]. Cells were surface-labelled with $Na^{125}I$ and lactoperoxidase and lysed in 1% NP40. The lysates were subjected to immunoprecipitation with the affinity-purified anti-γ antibody. As a negative control, the same lysates were immunoprecipitated with the anti-β-galactosidase antibody also affinity-purified from the same hyperimmune rabbit serum from which the anti-γ antibody was purified. The immunoprecipitate was analysed by SDS-PAGE under non-reducing conditions in one dimension and then under reducing conditions in the second. Under these conditions those cell-surface proteins containing interchain disulphide linkages migrate differently in the first and second dimensions and are usually displayed in the region below the diagonal line[15]. As shown in Fig. 4*a*, two off-diagonal spots of 45K and 35K are

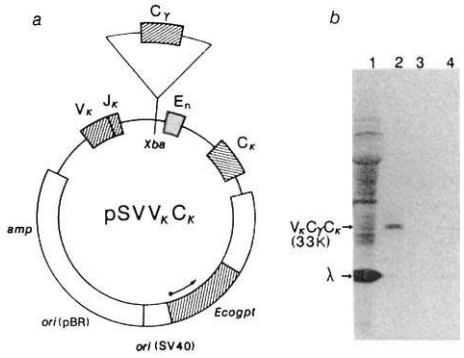

Fig. 3 Immunoprecipitation by the rabbit anti-γ antiserum of the $V_\kappa C_\gamma C_\kappa$ polypeptide chain secreted from a myeloma transfectant. *a*, Construction of the recombinant plasmid pSV $V_\kappa C_\kappa$ containing the C_γ exon within the major intron of a rearranged κ gene. The *Eco*RI–*Bam*HI DNA fragment containing the entire rearranged κ gene was cloned from a mouse myeloma LPC-1 and inserted to a plasmid pSV2gpt[26]. The *Hin*dIII DNA fragment containing major $C_{\gamma 2}$ exon[2] and its flanking sequences was then inserted at the unique *Xba*I site between the $V_\kappa J_\kappa$ exon and the enhancer (En). *b*, An SDS-PAGE autoradiogram of the immunoprecipitate of the $V_\kappa C_\gamma C_\kappa$ protein. ^{35}S-methionine-labelled supernatant of the culture of myeloma J558L stably transfected with pSV $V_\kappa C_\gamma C_\kappa$ (lane 1) was immunoprecipitated by goat anti-mouse κ light chains (lane 2), anti-β-galactosidase fraction (lane 3) or the anti-γ fraction of the rabbit serum obtained after hyperimmunization with the β-gal-γ fusion protein. J558L secretes a λ light chain in large amounts and some of it is carried over non-specifically in the immunoprecipitate.

Methods. A mouse myeloma J558L was transfected with pSV $V_\kappa C_\gamma C_\kappa$ by the protoplast fusion method as described previously[27]. The culture of stable transfectant D8.2 was fed with 0.4 mCi per ml ^{35}S-methionine (Amersham) for 5 h. The protein in the culture supernatant was denatured and partially renatured as described[20] and was subjected to immunoprecipitation and SDS-PAGE.

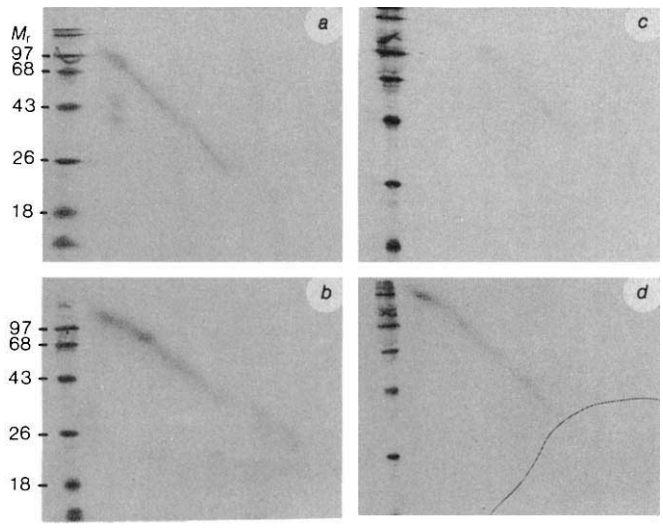

Fig. 4 Two-dimensional PAGE analysis of immunoprecipitated γ ^{125}I-labelled proteins from fetal thymocytes and CTL clone 2C. *a*, *b*, Lysates of fetal thymocytes from day 16 embryos immunoprecipitated by the anti-γ and anti-β-galactosidase fractions, respectively. *c*, *d*, Lysates of CTL clone 2C immunoprecipitated by anti-γ and anti-β-galactosidase, respectively. PAGE was performed under nonreducing conditions from left to right and reducing conditions from top to bottom.

Methods. Fetal thymi were dissected from the embryos of the day 16 pregnant mothers. The thymocytes were suspended in PBS and surface-labelled with ^{125}I using lactoperoxidase. The cells were washed three times with PBS, suspended in cold NP40 buffer (10 mM Tris-HCl *p*H 7.5, 0.1 M NaCl, 1 mM PMSF and 1% Nonidet P40) and disrupted by vigorous shaking. The lysates were subjected to immunoprecipitation and the immunoprecipitates were electrophoresed under nonreducing conditions through 10% acrylamide gels formed in a glass tube of internal diameter 1.5 mm. The gel was then removed from the glass tube, soaked in a buffer containing 5% β-mercaptoethanol for 30 min at room temperature, and placed on top of 12% slab gel for electrophoresis in the second dimension. The CTL clone 2C was analysed similarly.

present in the autoradiogram obtained with the lysate prepared from day 16 fetal thymocytes and the anti-γ antibody. No such spots were detectable when the anti β-galactosidase antibody was used (Fig. 4*b*).

The results of the analysis of the CTL clone 2C are shown in Fig. 4*d*. It seems that neither the 45K nor the 35K 'off-diagonal' component is present on the surface of this CTL clone.

The M_r of the $V_2J_2C_2$ γ-chain predicted by the nucleotide sequence is 33K and the chain should have no *N*-linked carbohydrate. Thus the measured molecular mass, 35K, of the smaller of the two 'off-diagonal' components observed in the fetal thymocytes is in good agreement with the predicted value. Preliminary experiments in which lysate was treated with dithiothreitol, immunoprecipitated and analysed by one-dimensional SDS-PAGE show a specific 35K component in the precipitates obtained with the anti-γ antibody which is competed out when excess purified β-gal-γ fusion protein is added to the lysate before immunoprecipitation (data not shown). Thus it seems that the thymocyte population derived from 16-day-old mouse fetuses contains cells that bear on the surface a γ-chain linked by one or more disulphide bonds to a 45K component.

As the anti-γ antibody was prepared by using as the immunogen the polypeptide encoded by a part of $V_2J_2C_2$ cDNA (see Fig. 1*a*), we tacitly assumed that the γ chain represented by the 35K component is of the $V_2J_2C_2$ type. But three more *V* gene segments, V_4, V_5 and V_6, are known to be expressed at least at the RNA level in fetal thymocytes in combination with another *JC* pair, J_1C_1[4,10,11]. Furthermore another γ gene, $V_1J_4C_4$, was recently reported[17]. As J_1C_1 and V_2 are highly homologous to J_2C_2 (94% homology at the protein level) and V_1 (87% homology), respectively, it is possible that the anti-$V_2J_2C_2$ antibodies used in this study crossreact with the γ-chain encoded by any of these four additional types of γ genes. Thus at least some of the 35K material shown in Fig. 4 may be non-$V_2J_2C_2$ γ chain.

The chemical nature of the 45K component is less clear. We cannot rule out the possibility that the 'off-diagonal' 45 K component observed in Fig. 4*a* carries a γ epitope. If this component is a γ-chain, its relative molecular mass seems to rule out the $V_2J_2C_2$ type. On the other hand, because any one of the other four known types of γ-chains may be *N*-glycosylated, its molecular mass could be as large as 45K. Another possible candidate for the 45K component is the β-chain; the β gene is known to be rearranged and activated for transcription by day 16 of fetal thymocytes[6–8]. A model of intrathymic development of T cells including thymocytes bearing a $\beta\gamma$ heterodimer has been presented[6,18,19], but no direct evidence for such cells has been obtained to date.

Yet another possible candidate for the 45K component is a product of a new gene referred to as δ. The existence of such a polypeptide is supported by recent work on human systems. Brenner *et al.* reported the identification of a putative second T-cell receptor present on the surface of a subpopulation of peripheral blood lymphocytes (PBL) from immunodeficient patients[20]. This protein is associated with the invariant protein CD3 (T3), and is composed of a 55K component reactive with anti-γ-peptide antibodies and a 40K polypeptide that does not react with these antibodies. Similarly Bank *et al.* reported a clone of normal immature $CD4^-8^-$ ($T4^-T8^-$) human thymocytes which bear CD3-associated dimer composed of anti-γ peptide-

reactive 44K component and anti-γ peptide non-reactive 62K component[21]. These authors propose that the component that does not react with the anti-γ peptide is a product of a new gene, δ, although its molecular mass is quite different (40K as against 62K) between the two publications.

The function of the cells bearing the putative $\gamma\delta$ heterodimer remains unknown. It has previously been reported that some CTL clones and many T helper (T_H) clones or hybridomas either lack γ RNA completely or contain only an out-of-phase γ RNA[5,9,10,22]. These data led some to conclude that the protein encoded by the reported γ genes is not an obligatory requirement for the function of these T cells. The present study shows further that even in 2C, a CTL clone in which the γ RNA is present and in-phase, the membrane expression of the γ polypeptide chain is absent and therefore not a prerequisite of the antigen-specific function of the T cell.

One possible view of the putative $\gamma\delta$ heterodimer is that it is 'isotypic' to the conventional $\alpha\beta$ TCR, in a relationship analogous to the κ and λ light chains in immunoglobulins. Indeed the structural resemblance among α, β and γ[23] is reminiscent of the similarity between κ and λ. Furthermore the $\gamma\delta$-bearing cells seem to have cytotoxic[21] or IL-2 producing capacity (D. Pardoll and A. M. Kruisbeek, personal communication). But, the apparent difference in the surface markers of the two types of T-lineage cells ($CD4^-8^-$ for $\gamma\delta$[20,21] and $CD4^+8^-$ or $CD4^-8^+$ for $\alpha\beta$) does not support this view. An alternative view of the $\gamma\delta$-bearing cells is that they define a T-cell subset other than CTL and T_H. A $\gamma\delta$-positive human cell line exhibiting a NK (natural killer) activity seems to exist[24].

As the putative $\gamma\delta$ heterodimer is expressed on the surface of the fetal thymocytes, one might also envision the possibility that such a structure could function as a receptor involved in the selection of a self MHC-restricted T-cell repertoire[25]. Before proposing a precise role of such a receptor in the expansion or deletion of specific thymocytes, however, several facts should first be established. These include the cell lineage relationship between the $\gamma\delta$- and $\alpha\beta$-bearing cells, the possible coexpression of the two receptors on the surface of a thymocyte subset and the functional ligands of the $\gamma\delta$ heterodimer.

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Note added in proof: Recently Lew *et al.* (*Science* **234**, 1401-1405; 1986) reported that a subpopulation of $CD4^-8^-$ adult thymocytes also express a γ-chain disulphide-bonded to a 45K component.

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A γ-chain complex forms a functional receptor on cloned human lymphocytes with natural killer-like activity

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We have recently derived from human fetal blood (25 wks) a series of cloned cell lines that were selected for their ability to kill the conventional natural killer (NK) target cell K562[1]. It was found that a fraction of these clones express CD3 proteins but not the monomorphic Ti $\alpha\beta$ determinant recognized by WT31 antibody[1]. One interleukin-2-dependent $CD3^+$ $WT31^-$ clone, termed F6C7, was used for immunization of mice to generate monoclonal antibodies directed at a potentially novel recognition receptor. It was shown that F6C7 cells, which transcribe Ti β but not Ti α genes, surface-express a clonotypic structure, termed NKFi[2]. Immunoprecipitations performed with anti-NKFi monoclonal antibody (mAb) indicated that the corresponding molecule is resolved in SDS-polyacrylamide gel electrophoresis (PAGE) as a single band of relative molecular mass ~85,000 (M_r~85K). After reduction, a major band was detected at 44K and a faint band was present at 41K[2]. The present study was designed to characterize this structure. It was found that NKFi represents either two 44K disulphide-linked γ (TCR) chains, or possibly one γ chain associated to an additional undetected molecule, and that the 41K material corresponds to a partially glycosylated fraction of the γ protein. Anti-NKFi mAb both induces a specific autocrine proliferative response and blocks cytotoxic function, demonstrating that γ chains serve as functional receptor structures on subpopulations of normal human lymphocytes.

In a first series of experiments, we assessed whether NKFi is a glycosylated protein. After immunoprecipitation with anti-NKFi mAb, a fraction of the material was treated with endo-F, an enzyme which cleaves both high-mannose and complex glycans[3,4]. Figure 1*a* depicts a typical SDS-PAGE analysis of NKFi under non-reduced conditions (lane A), reduced conditions (lane B) and reduced conditions following endo-F digestion (lane C). As shown, we usually detected two major deglycosylation products at 41K and 34K respectively. These results indicated the presence of *N*-linked glycans on the native 44K molecule. To establish whether the minor 41K native structure (containing generally one tenth of the total number of specific counts) was also glycosylated, each band was individually extracted from preparative gels. Separate electrophoresis of heavy (Fig. 1*b*, lanes A and B) and light (Fig. 1*b*, lanes C and D) material was then performed either directly (lane A and C) or after treatment with endo-F (lane B and D). These experiments indicated that the final digestion product of each band resolved at the same M_r of ~34K suggesting that the

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