anti-CD3, mAb 536, and mAb F23.1. The F23.1 antibody detects an epitope of the Vβ8 gene product and reacts with ~25% of mature α/β T cells[16]. As shown in Table 1, F23.1$^+$ cells were not detectable until day-17 of gestation and increased to a maximum fraction of CD3$^+$ cells by birth. Assuming that Vβ8 is expressed by 25% of α/β T cells at all stages of development, we used a multiplication factor of four to approximate the number of cells expressing α/β TCR in our analysis. The difference between total CD3$^+$ cells and the sum of Vγ3$^+$ and estimated α/β cells was used to estimate the fraction of cells that express γ/δ TCR using Vγ segments other than Vγ3. The data from Table 1 and the extrapolated values are presented graphically in Fig. 3 as a percentage of total CD3$^+$ cells (Fig. 3*a*), or as approximate numbers of cells (Fig. 3*b*) expressing Vγ3/δ, other γ/δ TCR, or α/β TCR as a function of gestational time.

It is apparent from Fig. 3 that there are at least four waves of appearance of cells bearing TCR in the developing thymus. The first to appear are cells that express Vγ3, which decrease steadily as a fraction of CD3$^+$ cells, being replaced by thymocytes bearing γ/δ TCR not encoded by the Vγ3 gene, and which comprise most of the CD3$^+$ cells on days 17 and 18 (Fig. 3*a*). Thymocytes bearing α/β TCR appear at day 17 and comprise most of the CD3$^+$ cells in the thymus after day 19 (Fig. 3*a*). Since the size of the thymus increases exponentially during fetal development, we also examined the contributions of each of the three classes of cells to the total number of cells (Fig. 3*b*). It is apparent that a third peak of cells expressing non-Vγ3 γ/δ TCR occurs at day 20 of gestation.

The data presented here demonstrate that the ordered expression of TCR genes[4,5,11] is reflected by the sequential appearance of distinct cell populations bearing the products of those genes during thymocyte development. It has been reported that γ- and δ-gene rearrangments during fetal ontogeny are not cumulative, suggesting the occurrence of a major change in the populations of cells present in the thymus[7]. Our data suggest that this change is a result of the death or emigration of the first wave of cells expressing TCR composed of the Vγ3 gene product. Although a lineal relationship has not been firmly established, it is reasonable to propose on the basis of our findings that this first wave of CD3$^+$ cells emigrate to seed the skin, giving rise to the Thy-1$^+$ dEC in adults. The fetal origin of Thy-1$^+$ dEC in the adult is supported by our observation (D. Asarnow *et al.*, unpublished results) that non-germ line encoded nucleotides are essentially absent from the junctions of the rearranged γ- and δ-genes of Thy-1$^+$ dEC clones; this is consistent with the absence of non-germ line encoded nucleotides from the junctions of rearranged δ-genes isolated from the fetal, but not from the adult, thymus[7,8]. It is interesting that the γ/δ TCR of Thy-1$^+$ dendritic cells occurring in intestinal epithelium[17] appear to use restricted Vγ and Vδ genes which are distinct from Vγ3 (C. Janeway, personal communication) and might be the descendants of the second wave of cells seen in the thymus. A mAb directed against the avian homologoue of the mammalian γ/δ TCR has recently been described[18]. In studies using this mAb, similar waves of TCR expression were seen in chicken embryonic thymus[18].

Taken together, our findings indicate that generation of components of the immune system is temporally programmed by the order of TCR gene rearrangement, and suggests that the periphery is seeded with discrete populations of T cells generated at different stages in development. It also appears that the capacity of the immune system to generate at least one class of cells, the dEC, is absent in the fully mature thymus. Whether this is due to changes in the thymic microenvironment or to alterations in the machinery of rearrangement remains to be determined.

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Lymphocyte activation by HIV-1 envelope glycoprotein

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Cell activation by phytohaemagglutinin, phorbol ester and by the supernatant of phytohaemagglutinin-stimulated peripheral blood mononuclear cells induces the expression and cytopathic effects of latent human immunodeficiency virus type-1 (HIV-1) *in vitro*[1-3]. The lymphocyte surface protein CD4 has been identified as a receptor for HIV-1[4,5] and binds the viral envelope glycoprotein (gp120)[6,7]. In the light of evidence indicating that one natural function of CD4 is as a growth factor receptor[8-10], we examined the ability of native gp120 to activate resting CD4-bearing lymphocytes. Our results indicate that gp120 has innate biological activity as a result of a specific interaction with CD4, inducing increases in intracellular levels of inositol trisphosphate and of calcium, and in interleukin-2 receptor expression and cell motility.

The CD4 protein, originally defined by monoclonal antibodies against antigens for T-lymphocyte membrane differentiation[11,12], has the capacity to alter multiple T-cell functions related to antigen recognition and signal transduction. There is good evidence that CD4 enhances T-cell activation by serving as a receptor for class II major histocompatibility (MHC) molecules present on stimulator or target cells[13-16]. Related studies[17-20] indicate that the presence of CD4 on the cell surface enhances the affinity of the T-cell antigen receptor for antigen, possibly by binding to class II MHC proteins on presenting cells. Structural considerations[21], and the internalization and phosphorylation of CD4 after phorbol ester or antigen stimulation[8,22,23], suggest that the CD4 protein may also function as a growth factor receptor, independent of class II MHC molecules[8-10]. In this context, we examined the ability of the CD4 ligand gp120 to stimulate intracytoplasmic signalling mechanisms and to activate resting CD4-bearing lymphoctyes.

Lymphocyte activation by antigen and anti-CD3 antibody acts through a common signal pathway with the generation of

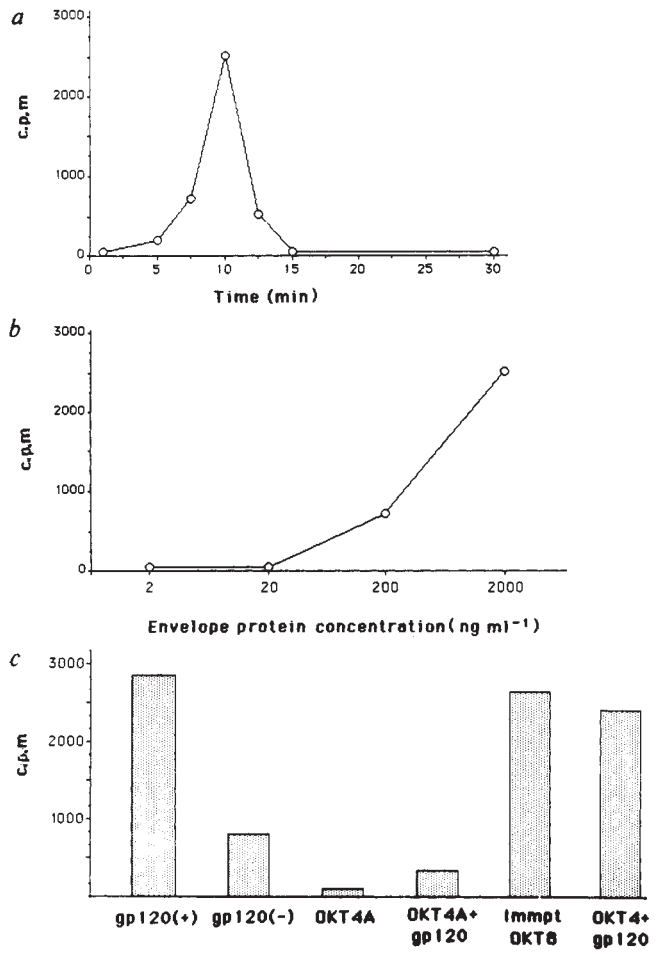

Fig. 1 The envelope glycoprotein of HIV-1 increases intracellular IP_3 levels. *a*, Time course of the gp120 effect on IP_3 levels. Myo[2-^{3}H] inositol-labelled T cells were incubated with 2.0 μg ml^{-1} envelope protein, corresponding to $\sim 10^{-8}$ M gp120. The reaction was stopped at the time points indicated and IP_3 measured as described below. *b*, Dose-response of the gp120 effect on IP_3. IP_3 levels were measured in cells incubated with different dilutions of gp120-containing supernatant for 10 min. *c*, Specificity of the gp120 effect on IP_3. IP_3 levels were measured after 10 min in cells incubated with 2.0 μg ml^{-1} gp120 (gp120(+)), gp120 immunoprecipitated with anti-gp120 (gp120(−)), or gp120 immunoprecipitated with OKT8 antibody (Immpt OKT8). In addition, cells were stimulated with OKT4A alone (OKT4A), or stimulated with gp120 following pre-incubation with OKT4A (OKT4A+gp120) or OKT4 (OKT4+gp120) antibody.

Methods. Nylon wool-nonadherent T cells were prepared from peripheral blood of HIV-1 seronegative volunteers[47] and incubated overnight in medium 199 with 0.4% bovine serum albumin. Cells were then incubated for three hours at 37 °C with 40 μCi myo[2-^{3}H]inositol per 20×10^6 cells. Aliquots of 20×10^6 cells were then incubated as above. At the indicated time points, inositol phosphates were isolated from cell lysates and identified by HPLC[48,49]. Results are expressed as (c.p.m. minus background) per 10^7 cells. Immunoprecipitation of gp120-containing viral supernatant was performed by binding goat anti-gp120 raised to acrylamide gel-purified gp120[31] or OKT8 antibody, to protein A-Sepharose CL-4B beads (Pharmacia) for 1 h at room temperature, washing, then incubating with gp120 to 1 h at room temperature.

inositol triphosphate (IP_3) from membrane phosphoinositides, which acts to release calcium from intracellular stores[24-29]. We therefore measured intracellular IP_3 and calcium levels in nylon wool non-adherent T cells prepared from peripheral blood of healthy HIV-1-seronegative donors after stimulation with gp120. The gp120 was prepared from the supernatant of HIV-1-infected H9 cell cultures by immunoaffinity chromatography[30]. The purified supernatant was ~50% gp120, as estimated by polyacrylamide gel electrophoresis and Western blotting. The results are expressed as the protein concentration of the purified supernatant (2.0 μg ml^{-1} corresponds to $\sim 1 \times 10^{-8}$ M gp120).

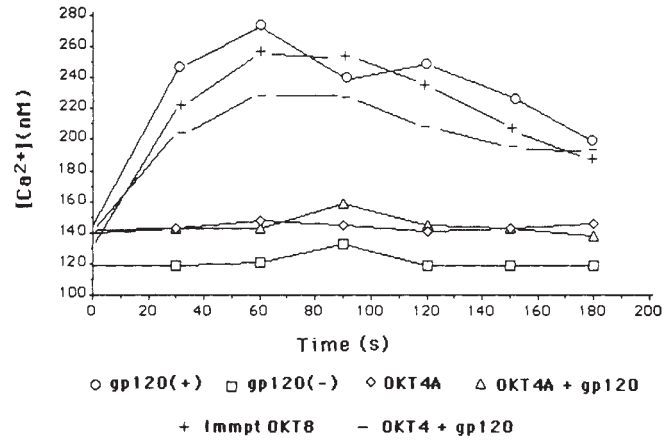

Fig. 2 The envelope glycoprotein of HIV-1 increases intracellular calcium levels. Indo-1-loaded T cells were exposed to 2.0 μg ml^{-1} gp120, gp120 immunoprecipitated with anti-gp120 sera or OKT8 antibody, OKT4A antibody alone, or pre-incubated with OKT4A or OKT4 antibody and exposed to gp120. Intracellular calcium was measured by fluorimetry.

Methods. Intracellular calcium levels were measured with the calcium probe indo-1. T cells were incubated in phosphate-buffered saline (*p*H 7.4) with 5 nM glucose and 5 μM indo-1 for 30 min 37 °C. Intracellular fluorescence was monitored by a Perkin-Elmer 650-10S spectrofluorimeter with an excitation wavelength of 355 nm, and emission wavelengths of 485 nm and 405 nm for calcium-free and calcium-bound signals respectively. Calcium concentrations were calculated as described[34].

For IP_3 measurements, cells were metabolically labelled with myo[2-^{3}H] inositol and [^{3}H]IP_3 was quantitated in cell lysate fractions eluted from an HPLC anion exchange column. As shown in Fig. 1*a*, gp120 caused a transient rise of IP_3, peaking at 10 min after stimulation. Increased IP_3 production occurred with 0.2 μg ml^{-1} gp120 and rose more than 10 times above basal levels at 2.0 μg ml^{-1} gp120 (Fig. 1*b*). Thus, this activity of gp120 is observed at concentrations close to the reported dissociation constant of recombinant gp120 for CD4 ($K_d = 4 \times 10^{-9}$ M; ref. 7). To demonstrate that the observed effect is due to gp120, immunoprecipitation was performed using goat anti-gp120[31] bound to protein A-coated Sepharose beads. The immunoprecipitated material had significantly reduced activity (Fig. 1*c*), whereas incubation with protein A alone, or protein A plus a neutral (OKT8) antibody did not reduce the activity of gp120. A preparation of partially purified p24 from HIV-1-infected H9 cells was also without activity in this system. Pre-incubation of cells with OKT4A antibody significantly inhibited the IP_3 response to gp120, but OKT4 had no blocking activity (Fig. 1*c*). This is consistent with competition between OKT4A, but not OKT4, and gp120 for CD4 binding[32,33]. None of the anti-CD4 antibodies alone stimulated IP_3 production.

Intracellular calcium ($[Ca^{2+}]_i$) measurements were conducted with the calcium probe indo-1 (ref. 34). As shown in Fig. 2, an increase in $[Ca^{2+}]_i$ from 140 to 270 nM occurred within 60 s of stimulation with 2.0 μ ml^{-1} gp120. The rise in calcium was reduced by ~15% if cells were stimulated in the presence of 1 mM EDTA, indicating that most of the calcium increase was due to release from intracellular stores. Neither anti-gp120-immunoprecipitated supernatant nor OKT4A induced a calcium response. Cells pre-incubated with OKT4A, but not with OKT4, failed to respond to gp120.

To determine whether the observed intracytoplasmic signals generated by gp120 were associated with activation of resting (G_0) T cells, we analysed interleukin-2 receptor (IL-2R) expression with fluorescent monoclonal anti-IL-2R antibody. Resting T cells express minimal levels of IL-2R and increased expression of this receptor occurs with progression from the G_0 to G_{1a} phase of the cell cycle[35]. We found that 2.0 μg ml^{-1} gp120 induced increased surface expression of IL-2R on resting T cells so that the percentage of positive cells rose from less than 5% to 18% at 24 h (Fig. 3*a*), reaching levels of up to 26% at 48 h (Fig. 3*b*). As with calcium and IP_3 measurements, the effects of gp120 were blocked by immunoprecipitation of the viral supernatant or by pre-incubation of cells with OKT4A antibody (Fig. 3*b*).

Experiments in our laboratory have demonstrated that T-lymphocyte growth factors interleukin-2 (IL-2), insulin, and insulin-like growth factor-1 (IGF-1) are all capable of inducing a motile response in lymphocytes[36–38], suggesting that this activity may be a general response of lymphocytes to growth factors. We therefore tested gp120 in chemotaxis experiments using a modified Boyden-chamber technique[39]. An increase in motility of more than 200% of the control was observed at gp120 concentrations similar to the IP_3 and IL-2R responses (Fig. 4*a*). A motile response was seen with T cells and enriched populations of CD4-bearing cells, but not with CD8-bearing cells, or if the gp120 was immunoprecipitated with anti-gp120 (Fig. 4*b*). Limited checkerboard analysis[40] indicated that the motile response to gp120 was chemotactic (directed), rather than chemokinetic (random) in nature (Table 1). The observation that gp120 induced a chemotactic response by CD4-bearing lymphocytes, suggests a possible mechanism for accelerating the destruction of uninfected CD4-bearing lymphocytes *in vivo*. Envelope protein shed from viable HIV-1-infected cells[41–43] (possibly monocytes/macrophages[44]) might recruit uninfected

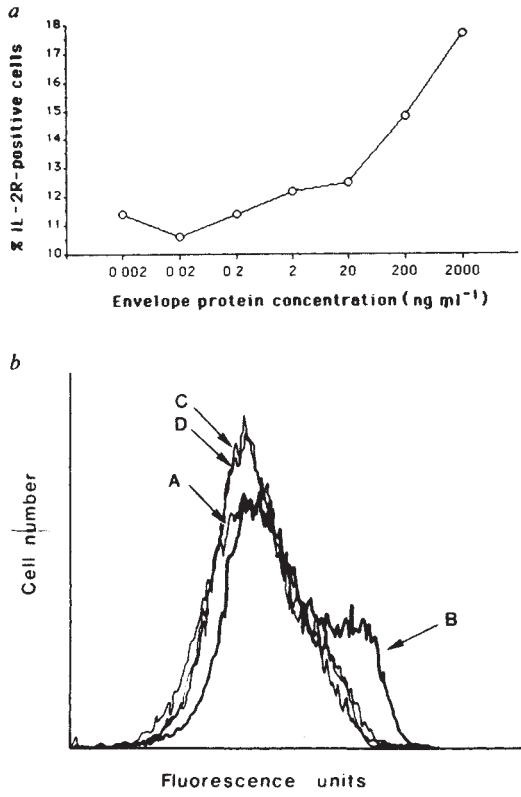

Fig. 3 The HIV-1 envelope glycoprotein induces increased expression of interleukin-2 receptors. *a*, Dose-response of the effect of gp120 on IL-2R expression. T cells were incubated with a range of gp120 dilutions for 24 h then stained with fluorescein-isothiocyanate-conjugated anti-human IL-2 receptor (CD25, Becton-Dickinson) monoclonal antibody and analysed with a Becton-Dickinson FACS 440 as described[10]. Less than 5% of cells incubated in medium alone express detectable levels of IL-2R *b*, Specificity of the effect of gp120 on IL-2R expression. T cells were incubated in control buffer (*A*), 2.0 μg ml^{-1} envelope protein (*B*), anti-gp120 immunoprecipitated viral supernatant (*C*), or pre-incubated with OKT4A monoclonal antibody (Ortho) for 1 h and washed before exposure to gp120 (*D*). After 48 h cells were stained with fluoresceinisothiocyanate-conjugated anti-IL-2R antibody and analysed as above. The shift in IL-2R expression represents an average increase from less than 5% to 26% IL-2R-bearing cells. IL-2R expression in cells incubated with viral supernatant that was immunoprecipitated with anti-gp120 or cells pre-incubated with OKT4A was less than 6%. Immunoprecipitation with OKT8 antibody, or pre-treating cells with OKT4 antibody before gp120 stimulation resulted in 21% and 26% IL-2R-bearing cells respectively.

Table 1 Checkboard analysis of gp120

		Envelope protein concentration above filter (μg ml^{-1})			
		0	0.2	1.0	2.0
Envelope protein concentration below filter (μg ml^{-1})	0	100%	127 ± 21%	104 ± 15%	72 ± 18%
	0.2	158 ± 24%*	131 ± 18%		
	1.0	208 ± 19%*		119 ± 17%	
	2.0	255 ± 24%*			102 ± 21%

T cells were analysed in the Boyden chamber for motility in response to three dilutions of gp120 added above, below, or above and below the filter as indicated. Results are expressed as percentage ± standard deviation of migration in control buffer alone. Migration in control buffer was 8.0 ± 1.1 cells per high power field.
* Significant difference ($P < 0.05$) between experimental sample and control by analysis of variance.

CD4-bearing lymphocytes from the circulation to sites where they could be destroyed by syncytium formation or other mechanisms, just as chemoattractant cytokines recruit cells to foci of inflammation.

Our data indicate that binding of gp120 to CD4 activates the IP_3 and calcium signal mechanism in resting T cells, inducing the increased expression of IL-2R and a chemotactic motile response. These results support the hypothesis that CD4 can function as a signal-transducing receptor for a growth factor ligand, and indicate that gp120 can serve as a useful probe for studying normal functions of CD4. A candidate CD4-binding lymphokine, lymphocyte chemoattractant factor induces similar activities in CD4-bearing lymphocytes and monocytes[10,37,39].

Cell activation by lectins, phorbol and cytokines have all been found to stimulate virus expression from cells latently infected with HIV-1 (refs 1–3). Although the significance of gp120-induced lymphocyte activation in the pathogenesis of HIV-1 infection remains to be determined, it is possible that signals generated by gp120 binding might activate the target cell to provide a receptive environment for subsequent steps in the infection process or contribute to the expression of cytopathic effects. A common mechanism for this phenomenon could be the induction of cellular transcription factors capable of activating the HIV-1 promoter[45,46]. Although T-cell activation by envelope protein may be a significant factor in the pathogenesis of HIV-1 infection, there is no evidence for this at present. The implications of innate biological activities of gp120 should be considered in the design and implementation of vaccine studies using this protein.

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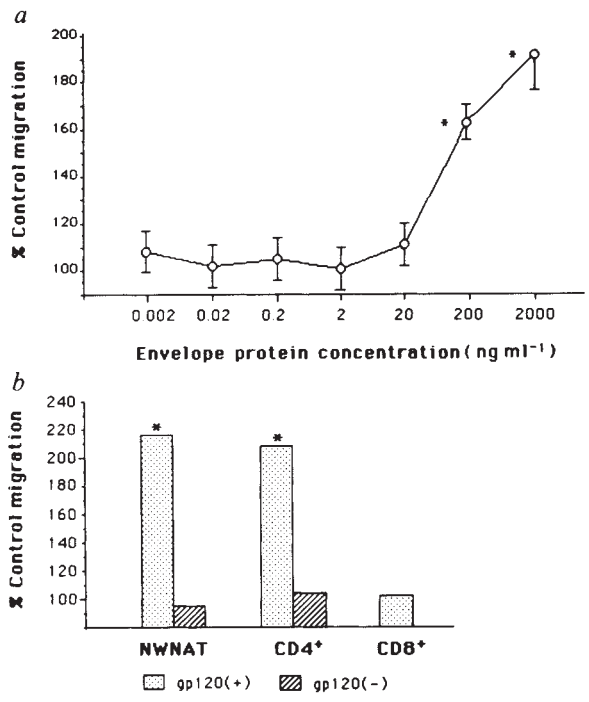

Fig. 4 Chemoattractant activity of HIV-1 envelope glycoprotein *a*, Dose-response of the effect of gp120 on T-lymphocyte motility. T cells were incubated with varying dilutions of gp120 and the motile response was quantitated in a modified Boyden chamber assay[39]. Results are expressed as a percentage in a modified Boyden chamber assay[39]. Results are expressed as a percentage of migration of unstimulated control cells ± standard deviation, *b*, Specificity of the effect of gp120 on lymphocyte motility. Nylon wool-nonadherent T cells, enriched populations of CD4-bearing cells, and enriched populations of CD8-bearing cells were stimulated with $\sim 10^{-8}$ M gp120 and analysed in the Boyden chamber assay. Stippled bars represent the response to gp120 whereas hatched bars represent the response to viral supernatant immunoprecipitated with anti-gp120 serum. *Significant difference ($P < 0.05$) between experimental sample and control by analysis of variance. **Methods.** T cells were suspended in M-199 containing 0.4% bovine serum albumin at 10×10^6 cells ml^{-1}. Enriched populations of CD4-bearing and CD8-bearing cells were prepared by negative selection as described[37]. This procedure yields cells which are >95% CD4- or CD8-bearing. Chemotaxis chambers were prepared using 8-μm pore nitrocellulose filters separating buffer control or experimental samples in the lower wells from cells in the upper chamber. Migration was quantitated by counting total cells moving in the filter beyond a depth of 40 μm. Migration in buffer control in these experiments ranged from 6.6 ± 1.3 to 10.7 ± 1.5 cells per high power field.

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Structure and function of human perforin

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Perforin (P1) is a cytolytic protein with similarity to complement component C9. P1 has been described as a unique component of murine cytolytic T-cell and rat natural killer cell granules[1,2]. Previous studies[3,4] indicated that human granules and P1 differed from murine granules and P1 in that they appeared to be cytolytically less active and lacked the haemolytic activity characteristic of P1. It has been suggested that P1, like C9, is under the control of the homologous restriction factor[5]. Here we determine the primary structure of human P1, re-examine its functional properties, and address the question of homologous restriction.

Figure 1*a* shows the complementary DNA and derived amino-acid sequence of human P1. Figure 1*b* shows the homology of human P1 with the human complement proteins forming the transmembrane channel of the membrane-attack complex. The overall homology in the aligned stretch is ~21% for C8α, 19% for C7 and 17% for C8β and C9. Only ~370 of the 534 amino acids, spanning the putative membrane-binding region and the cysteine-rich epidermal growth factor-type domain, are