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Academic Dissertation

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SYNTHETIC PEPTIDES AS PROBES FOR PROTEIN INTERACTIONS AND AS ANTIGENIC EPITOPES

Ale Närvänen

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In this study synthetic peptides have been used both as antigens in immunological studies, as well as probes in a study of protein interactions.

Rabbit antibodies to the synthetic peptide sp23 derived from a cloned sequence of human endogenous retroviral gene erv-1 were used in a search for the expression products in human cell cultures and tissues. The cross-reactivity of the anti-peptide antibodies led to the detection of a novel protein from human choriocarcinoma cell line. This protein of Mr 75 000 was purified by using conventional biochemical methods and the purification steps were monitored with anti-sp23 antibodies.

Synthetic peptide deduced from the cDNA sequence of the transmembrane protein gp41 of human immunodeficiency virus HIV-1, the causative agent of AIDS, have been used as antigen detecting human antibodies to HIV-1. In clinical studies, strong antibody responses against this peptide were found during the early stages of HIV infection, including cases with seroconversion. The antibody level tended to decrease in the sera at the ARC and AIDS stages of HIV infection.

Human antibodies to a synthetic peptide containing this sequence were affinity-purified and tested using various immunological methods. These affinity-purified antibodies did not react with the gp41 protein, suggesting that this epitope is partially or totally buried in the molecule and may only be exposed during antigen processing in vivo in the HIV-infected individuals.

Laminin is a 1000 kd extracellular matrix protein, that has a multidomain structure with various functions such as neuronal cell-adhesion and the promotion of brain development and neurite outgrowth. In studies to locate functional regions in laminin molecule, synthetic peptides have been used in biological studies in vitro. A synthetic peptide derived from the maximally amphipathic region in the carboxy terminal part of B2 chain of laminin showed neurite outgrowth-promoting activity in neuronal cell cultures.

Key words: Synthetic peptides; anti-peptide antibodies; HIV-1; laminin; cytovillin

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Contents

List of **original publications**

This thesis is based on the following publications. The papers are referred to in the text by their Roman numerals (I-IV). Some unpublished data are also included.

I. Närvänen, A 1985: Purification, from cultured choriocarcinoma cells, of a 75000-Mr protein reacting with antibodies to a synthetic peptide based on a cloned human endogenous provirus nucleotide sequence.-Biochem.J. 231:53-57.

II. Närvänen A., Korkolainen, M., Kontio, S., Suni, J., Turtiainen, S., Partanen, P., Soós, J., Vaheri, A., & Huhtala, M-L. 1988: Highly immunoreactive antigenic site in a hydrophobic domain of HIV-1 gp41 which remains undetectable with conventional immunochemical methods.-AIDS 2:119-123.

III. Närvänen, A., Korkolainen, M., Suni, J. Korpela, J., Kontio, S., Partanen, P., Vaheri, A. & Huhtala, M-L. 1988: Synthetic env gp41 peptide as a sensitive and specific diagnostic reagent in different stages of human immunodeficiency virus type 1 infection.-J.Med.Virol. 26:111-118.

IV. Liesi, P., Närvänen, A., Soós, J., Sariola, H. & Snounou, G. 1989: Identification of a neurite outgrowth-promoting domain of laminin using synthetic peptides.-FEBS Lett. 244: 141-148.

Abbreviations

AIDS	Acquired Immuno Deficiency Virus
ARC	AIDS-related complex
ASX	asymptomatic HIV-1 positive patient
BSA	bovine serum albumin
DCC	dicyclohexylcarbodiimido
DCM	dichloromethane
DMF	dimethyl formaminde
DRG	dorsal root ganglia
EIA	enzymeimmune assay
EIU	enzymeimmune unit
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HOBt	hydroxybenzotriazole
Ig	Immunoglobulin
LAS	lymphadenopathy syndrome
MBS	m-maleimidobenzoic acid N-hydroxysuccinimide ester
MEM	Eagle's minimal essential medium
MeOH	methanol
PBS	phosphate buffered saline
RIPA	radioimmune precipitation assay
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel-electrophoresis
SPPS	Solid phase peptide synthesis
t-Boc	tert-butyloxycarbonyl
TFA	trifluoroacetic acid

One-letter abbreviations of amino acids

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

1. Introduction

1.1. Epitope predictions with computer analyses

One of the basic problems in modern molecular biology is to elucidate the correlation of the biological functions of proteins to their structure. Numerous attempts have been made to resolve the structure of different proteins from their primary structure as well as to relate the different predicted structures and physicochemical features to the functions of the proteins and polypeptides.

1.1.1. Secondary structures

The most notable method for predicting three-dimensional aspects from the amino acid sequence alone is the prediction of local secondary structures. This is based on empirical methods, in that they utilize a library of known protein structures. According to the frequency with which the individual amino acids or short peptides occur in alpha-helices, beta-sheets or reverse turns, any new sequence can be systematically scanned and the probability of occurence of secondary structures can be evaluated (Chou & Fassman 1973, Wu & Kabat 1973, Garnier et al. 1978).

Computerized programs have been developed on the basis of these frequencies. Deleage and coworkers have tested the original scheme of Chou and Fasman. The percentage of correctly predicted amino acids was between 41 and 66% for a three-state (helix, sheet and coil) description of protein secondary structure (Deleage et al. 1987).

A more developed method for protein secondary structure prediction is based on a neural network. In this method known secondary structures and amino acid sequences are used to teach the network. Using a set of 48 proteins with known structure as a reference, a set of 14 proteins were analysed, and the method achieved on overall predictive accuracy of 63%. When predictions were filtered to include only the strongest 31% of predictions, the predictive accuracy rose to 79% (Holley & Karplus 1989).

A pattern-matching approach for prediction of beta-turns in proteins has achieved high accuracy (95%) on a test set of proteins of known structure. Applying the same procedure to homologous protein families gives a 90% success rate (Cohen et al. 1986). According to these methods, the highest accuracy is reached when the secondary structures of the proteins within the same protein family are predicted, and a proper tertiary structure data base is available.

1.1.2. Hydrophobicity

According to empirical observations, hydrophobic amino acid side chains tend to be buried within the native structure of proteins (Chotia 1976, Rose et al. 1985). The hydrophobicity can thus be used to distinguish regions of interior sequences from regions of exterior sequences (Chotia 1984). The distribution of hydrophobicity over the amino acid sequence can be calculated using the hydrophobicity values of all the individual amino acids. In Table 1 there are three hydrophobicity indices for each of the 20 essential amino acids. Hydrophobicity is calculated along the polypeptide, averaging hydrophobicity parameters over segments ("windows") of different lengths. Commonly used "windows" consists of 5 to 11 amino acid residues for calculations. For transmembrane studies, "a window" of 20 amino acids is used.

Table 1. Three different hydropathy indices for the 20 essential amino acids: Consensus (Eisenberg 1984b), K&D (Kyte & Doolittle, 1982), H&W (Hopp & Woods, 1981). In Consensus and K&D indices positive values indicate hydrophobic side chains and negative values hydrophilic side chains. In H&W the index values are vice versa.

	Consensus	K&D	H&W
Isoleucine	1.40	4.5	-1.8
Phenylalanine	1.20	2.8	-2.5
Valine	1.10	4.2	-1.5
Leucine	1.10	3.8	-1.8
Tryptophane	0.81	-0.9	-3.4
Methionine	0.64	1.9	-1.3
Alanine	0.62	1.8	-0.5
Glycine	0.48	-0.4	0.0
Cysteine	0.29	2.5	-1.0
Tyrosine	0.26	-1.3	-2.3
Proline	0.12	-1.6	-1.4
Threonine	-0.05	-0.7	-0.4
Serine	-0.18	-0.8	0.3
Histidine	-0.40	-3.2	-0.5
Glutamic acid	-0.74	-3.5	2.5
Asparagine	-0.78	-3.5	0.2
Glutamine	-0.85	-3.5	0.2
Aspartic acid	-0.90	-3.5	2.5
Lysine	-1.50	-3.9	3.0
Arginine	-2.50	-4.5	3.0

1.1.3.Amphiphilicity

A special feature that alpha-helices tend to have, on average, is a strong periodicity in the hydrophobicity of 3.6 residues, the period of the alpha-helix. The segments of alpha helix can be amphiphilic, in the sense that one side is more apolar than the other (Eisenberg et al. 1982). The amphiphilicity of the protein segment, the hydrophobic moment, can be calculated from the relationship in this equation

$$\mu = \sum_{n=1}^{N} H_n S_n$$

in which Hn is the numerical hydrophobicity of the nth residue and S_n is a unit vector in the direction from the nucleus of the alpha-carbon toward the geometric center of the side chain. Pro-

tein segments with most apolar side chains on one side are characterized by large values of μs (Eisenberg et al. 1984a).

The strip-of-helix hydrophobicity algorithm can identify alpha or 3/10 helix structures having one axial, hydrophobic strip and otherwise variably hydrophilic residues. The mean of the hydrophobicity of amino acids in such a strip is calculated (Reyes et al. 1989).

1.1.4. Flexibility

The flexibility calculations are based on temperature factors obtained from x-ray crystallographic studies. Flexible, "hot regions" are located on the surface of the protein whereas rigid, "cold regions" are more likely to be buried in the molecule (Tainer et al. 1984). To calculate the local chain flexibility the temperature factors, i.e. B-values of the alpha-carbon atoms from 31 proteins, have been used as a data base. These values have been used to average the amino acid sequence of the protein studied (Karplus & Schultz, 1985).

1.1.5. Correlation between predicted structure and function

There are no rules for a direct correlation between the predicted structure of a certain area of a given protein and its biological activity. A number of calculations have been made in order to study if a predicted secondary structure of a protein also includes biological activity.

Thus, e.g. analysis of the hydrophobicity of the acetylcholine receptor subunit sequences reveals regions of amphipathic secondary structure. The amphipathicity peaks in the highly conserved region that Noda et al. postulate probably contains the acetylecholine-binding sites in the receptor alpha-subunits (Noda et al. 1982, Finer-Moore et al. 1984).

The mammalian G-proteins provide another example. These proteins transduce information from extracellular signals, including

neurotransmitters, hormones and sensory stimuli, into regulation of effector enzymes or ion channels within cells. The extreme carboxy-terminus of the alpha-chain of G-protein has been predicted to have a high amphipathic alpha-helix probability (Masters et al. 1986). This region has been shown to make direct contact with its receptors (Sullivan et al. 1987).

1.1.6. Predictions of antigenicity

Antigenic determinants may be either discontinuous or continuous. Discontinuous epitopes are conformation dependent and the amino acids which are involved in antibody binding are distant in the primary structure of that protein. A continuous epitope consists of a linear sequence of amino acids (Lerner 1982). The predictions for the location of continuous epitopes have been used in raising antibody probes specific for proteins, by immunization with synthetic peptides (Walter & Doolittle, 1983), as well as to develop synthetic peptide vaccines (Berzofsky 1985, Westhof et al. 1984, Steward & Howard 1987, Dalgleish et al. 1988). The idea of this approach is to predict surface areas of the proteins where antibodies can bind and probably block the active site of the protein. From the studies of well-characterized proteins such as myoglobin, lysozyme and cytochrome c, the primary reason why certain polypeptide chain segments are antigenic has been tentatively attributed to their exceptional surface exposure (Novotn'y et al. 1986, Krchnak et al. 1987). Several methods have used to predict antigenic regions from the primary structure of a protein.

Thornton and coworkers have constructed a method to approximate which amino acid side chains protrude from the surface of the protein. The "protrusion index" for each amino acid has calculated according to location of an amino acid in the ellipsoid structures of the globular protein (Thornton et al. 1986). Segmental hydrophilicity has been found to correlate with antigenicity only when the highest peak has been tested (Hopp 1986), but if all the known continuous epitopes in well-defined proteins were analyzed the correlation is not good (Westhof et al. 1984, Thornton et al. 1986, Geysen et al. 1987).

Pfaff and coworkers have selected a peptide sequence according to amphipathicity from the VP1 of foot-and-mouth disease virus. Rabbit antibodies raised to a synthetic peptide from the region 144-159 were able to neutralize the virus (Pfaff et al. 1982). Correlations between high flexibility and antigenic epitopes have been found in tobacco mosaic virus protein, myoglobin and lysozyme (Westhof et al. 1984) as well as in insulin and cytochrome c (Tainer et al. 1984).

Studies with monoclonal antibodies against peptide sequences in the alpha-helical region of myoerythrin have shown that both highly exposed, hydrophilic residues and buried, hydrophobic residues are important in antibody binding (Geysen et al. 1985, Getzhof et al. 1987). Two of the epitopes, residues 69-73 and residues 79-84, were amphipathic in nature. In these epitopes replacement of exposed and buried amino acids in corresponding synthetic peptide abrogates antibody binding, suggesting their direct involvement in the formation of the antigen-antibody interface (Fieser et al. 1987).

1.2. Antigenic epitopes in natural infection

During viral infection, viral proteins can induce both B cell and T cell responses. B cells interact directly with an antigen and recognize epitopes which are dependent on the native structure of a protein. T-cells activate cellular immunity and cytotoxic T lymphocytes. T cells are unable to recognize the antigens without the help of major histocompatibility complex (MCH) class I or class II molecules (Acuto & Reinherz 1985). T cells do not discriminate between native and denatured proteins. It has been shown that the majority of T cells do not recognize the native conformation of protein antigens (Unanue 1984, Schwartz 1985), but bind to denatured antigen (Streicher et al. 1984) or linear peptide fragments of the antigen (Townsend et al. 1986). To generate a T-cell response most antigens have to be in conjuction with MHC antigens. Ia receptors are located on the surface of the accessory cells, macrophages. These cells are involved in antigen processing and presentation to T cells. During processing, the accesory cells enzymatically fragment the antig-

en and present it together with the Ia receptor to the T cells (Allen 1987, Delovitch et al. 1988).

The studies with synthetic peptides have shown that Ia molecules show a very broad specificity. Some residues appear to interact both with Ia and with T-cells, leading to a model in which a peptide antigen is "sandwiched" between Ia and T-cell receptor (Sette et al. 1987). The minimal antigenic determinant for MHC class I restricted T lymphocytes is limited to a pentapeptide segment (Reddehase et al. 1989). In studies on the three dimensional structure of the HLA-A2 receptor it has been proposed that this receptor has special affinity to alpha-helical structures (Björkman et al. 1987) especially if the helix has an amphipathic feature (Delisi & Berzofsky 1985).

During antigen processing, a protein loses its original three-dimensional structure (Reyes et al. 1989). This makes it difficult to predict all naturally occuring linear epitopes according to surface or secondary structure calculations of the intact protein. In the transmembrane protein gp41 of human immunodeficiency virus type I (HIV-1) we and others have defined a highly immunodominant linear epitope. This epitope is highly hydrophobic and the flexibility probability is very low (Huhtala et al. 1987, Modrow et al. 1987)

1.3. Anti-peptide antibodies as probes for proteins

Antibodies to synthetic peptides derived both from nucleotide and amino acid sequences have been used to detect and study proteins which are difficult to purify or for which only the corresponding nucleotide sequence is known (Walter & Doolittle 1983). Anti-peptide antibodies are directed to short segments of amino acids, and the reactivity of these antibodies with target protein is based on cross-reactivity.

A good example of using antibodies against synthetic peptides as probes for proteins is illustrated by the discovery of cytovillin.

Endogenous retroviruses have been found to be present as multiple DNA copies in the genome of many vertebrates. The retroviral sequences are transmitted vertically as Mendelian genes. By using known primate endogenous retroviruses as probes, these proviruses were originally characterized also in the human genome. One of the sequences was cloned by using an endogenous chimpanzee retroviral pol gene as a probe. This probe is highly related to baboon endogenous retrovirus (BaEV). The cloned human sequence termed HC-20 (or endogenous retrovirus-1;erv-1) contains gag and pol genes, which are significantly related to these of both Moloney murine leukemia virus (Mo-MuLV) and BaEV (Bonner et al., 1982). A decapeptide with an additional cysteine in the aminoterminus (CENPSQFYERL) was deduced and synthesized from this proviral sequence at locus erv-1. This sequence has homology with MuLV and BaEV p30 proteins (Suni et al. 1984). Rabbit antibodies to this peptide were raised. These antibodies detected, on immunoblotting, a 75000-Mr protein both in first trimester human placental syncytiotrophoblasts and in cultured choriocarcinoma cells (Suni et al. 1984). In addition, anti-peptide antibodies detected a 75 000 Mr polypeptide in human renal cell adenocarcinoma (hypernephroma) tumor tissues (Wahlström et al. 1985).

Polyclonal antibodies raised against the purified Mr 75 000 polypeptide reacted with cultured choriocarcinoma cells (JEG-3) at light and electron microscopic levels with a protein which had highly restricted distribution in microvilli on the apical cell surfaces (Pakkanen et al. 1987). This protein, later designated as cytovillin, was found to be expressed in a wide variety of normal and transformed human cell lines and to be restricted to cell surface extensions, including microvilli, blebs, and long cell surface protrusions (Pakkanen 1988). More recently cytovillin was cloned, and computer analysis of the cytovillin cDNA sequence made from EMBL DNA and protein data banks revealed no significant homology to other DNA and protein sequences (Turunen et al. 1989).

Monoclonal antibodies to synthetic peptides can also express a wide cross-reactivity between various proteins. This may reflect similarities in the amino acid sequence or similar physicochemi-

cal properties of the immunogenic peptide and the epitopes of the target proteins.

Monoclonal antibodies to a synthetic peptide of six amino acids derived from the carboxy terminus of the transforming protein of Rous sarcoma virus p60src recognize the corresponding protein in transformed cells. However, these monoclonal antibodies also react with a number of constituents of untransformed cells. The reactivity of the monoclonal antibody with proteins identified as myosin, tubulin, vimentin and an unknown intracellular antigen were inhibited by the peptide. (Nigg et al. 1982).

A monoclonal antibody against a synthetic peptide SEDYGKDL, derived from a conserved sequence in the chicken alpha-fodrin repeats reacted in immunoblotting with avian alpha-spectrin and alpha-fodrin, both mammalian spectrins and with mammalian alpha-fodrin. This monoclonal antibody also reacted with alpha-actinin in both chicken and human cells. Only partial sequence homology was found between SEDYGKDL and chicken alpha-actinin or chicken alpha-fodrin (Närvänen et al. 1987) (Fig. 1).

1	O	R	W	K	P	L	L	A	S	E	D	Y	G	K	V	L	A	S	D	N	N	L	L	K	K	H
2	L	I	H	R	H	R	P	E	L	I	D	Y	G	K	L	R	K	D	D	P	L	T	N	L	N	T
3	D	N	Q	E	N	E	Q	L	M	E	D	Y	E	K	L	A	S	D	L	L	E	W	I	R	R	T

Figure 1. Two proposed antibody-binding sites on chicken alpha-actinin (amino acids 121-147, row 2 and 208-233, row 3) corresponding to chicken alpha-fodrin (amino acids 6-31, row 1). Putative antigenic determinants are boxed.

Polyclonal anti-virus antibodies have been demonstrated to be more specific than anti-peptide antibodies. Antisera to three synthetic peptides derived from three different strains of foot-and-mouth disease virus (Fig. 2) have been compared to corresponding anti-virus antibodies. As shown in table 2 anti-virus

antibodies showed lower crossreactivity with peptides derived from two other strains than anti-peptide antibodies (Geysen et al. 1985).

FMDV, type O1	G D L Q V L A K
FMDV, type A10	G D L G S I A K
FMDV, type C1	D L A H L T A K

Figure 2. The sequences of the synthetic peptides derived from three subtypes of foot-and-mouth virus.

Table 2. Antibody-binding activity of anti-peptide and anti-virus sera in peptide EIA. Activity of the sera is shown as the value obtained for the heterologous reaction expressed as a percentage of that for the homologous reaction.

	peptide		
Serum tested	O1	A10	C1
Anti-peptide serum			
peptide O1	100	30	73
peptide A10	<10	100	10
peptide C1	87	49	100
Anti-virus serum			
Type O1	100	<10	<10
Type A10	<10	100	<10
Type C1	10	19	100

The specificity of the anti-peptide and anti-virus antibodies were compared by testing which amino acid residues in each epitope are important for the immunoreactivity (Fig. 3).
A replacement set was synthesized, consisting of all of the peptides derived by substituting one residue at a time, all 19 alternative amino acids at each position of the parental sequence. Anti-peptide sera were found to be less specific than anti-virus antibodies; more amino acid substitutions were allowed using antipeptide antibodies than anti-virus antibodies (Geysen et al. 1985).

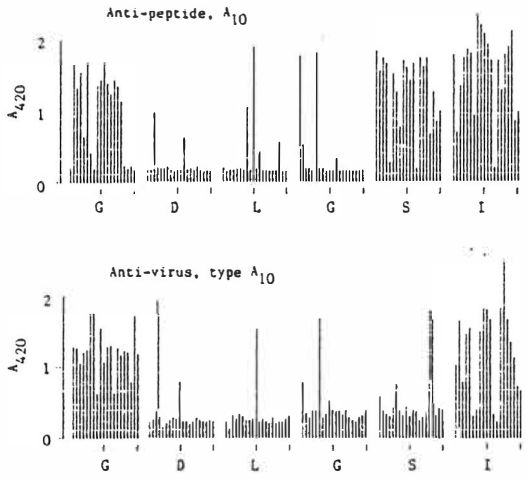

Figure 3. The antibody-binding activity for each peptide is shown as a vertical line proportional to the EIA absorbancy obtained. Every group of 20 lines corresponds to the complete replacement set for one of the six amino acid positions in the hexapeptide (Geysen et al. 1985)

1.4. Synthetic peptides in HIV serology

1.4.1. HI-viruses type 1 and 2

Acquired immunodeficiency syndrome (AIDS) was first described in 1981 (Centers of Disease Control 1981). This disease is manifested by opportunistic infections, predominantly pneumonia caused by Pneumocystis carinii, and certain forms of cancer, in particular Kaposi's sarcoma and brain lymphomas and disorders of the central nervous system. Before development of a full-borne AIDS with clinical manifestation of the disease, pre-AIDS is frequently noticeable, characterized by chronic lymphoadenopathy or leukopenia involving helper T lymphcytes, leading to the progressive immunodeficiency. In the AIDS phase, absolute lymphopenia and reduction of subpopulations of helper T lymphocytes occur (OKT4+;CD4+) (Gold et al. 1982, Hanrahan et al. 1982, Centers of Disease Control 1982).

The etiological agent of AIDS was first described in 1983 (Barre-Sinoussi et al. 1983). The virus, isolated from the peripheral lymphocytes of AIDS patients, is a lentivirus, a member of the Retrovidae family (RNA TUMOR VIRUSES; Fig. 4). This virus was subsequently isolated in several laboratories, and was vari-

ously referred to as Lymphoadenopathy virus (LAV), HTLV III and the AIDS-associated retrovirus ARV (Barre-Sinoussi et al. 1983, Popovic et al. 1984, Levy et al. 1984). Later all these viruses were renamed as human immunodeficiency virus 1 (HIV-1) by an International Committee On Taxonomy of Viruses (Nature 321,10: 1986).

During the retrovirus infection, reverse transcriptase produces dsDNA from viral ssRNA. Viral DNA then integrates into the host cell genome as provirus. Provirus can produce viral proteins using the protein synthesis machinery of the host cell. Viral proteins are coded as large polyproteins (Fig. 5) and are processed from the precursor proteins during the post-transcriptional maturation of the viral particle.

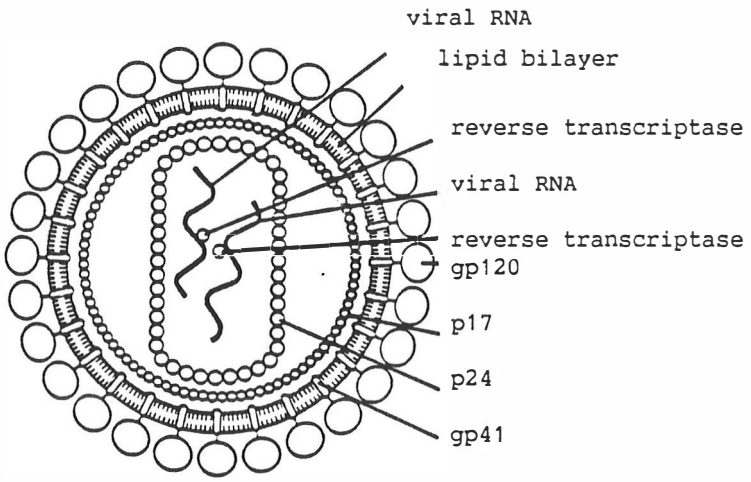

Figure 4. Schematic representation of HIV-1 virus particle. Viral ssRNA and reverse transcriptase are packaged into a core. Core consists of a major structural protein and a outer core protein. Virus particles have a lipid envelope, where envelope glycoproteins are anchored in the bilayer (Coulis et al. 1987).

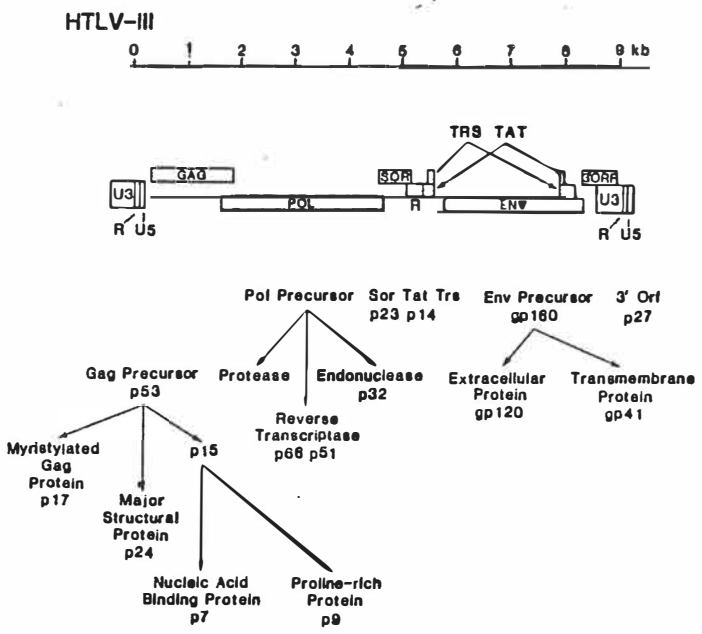

Figure 5. Genomic organization of HIV-1. Precursor proteins are encoded by three genes. Gag encodes core proteins, pol functional proteins and env envelope proteins (Human Retroviruses and AIDS 1988).

In 1986 another retrovirus was isolated from the AIDS patients in West-Africa (Clavel et al. 1986). This virus, named HIV-2, shares with HIV-1 its morphology, lymphotropism and cytopathic effect, but differs in its genome structure from HIV-1 (Brun-Vezinet et al. 1987, Guyader et al. 1987). Simian immunodeficiency viruses (SIVs) constitute a group of primate retroviruses that are morphologically and antigenically related to human immunodeficiency viruses (Henderson et al. 1988). SIV belongs to a group of cytopathic retroviruses, whose prototype STLV-3mac was identified in captive rhesus monkeys (Macaca mulatta) with an AIDS-like disease (Daniel et al. 1985). According to nucleotide sequence homology analyses, 83% of predicted amino acids of Simian Immunodeficiency Virus SIV_{Mne} were homologous to HIV-2, and 41% were identical to the predicted residues of HIV-1 (Henderson et al. 1988).

Studies at the molecular level have revealed that a high degree of genetic polymorphism exists among HIV-1 and HIV-2 viruses. HIV-1 isolates in North-America and Western Europe are more closely related to each other than with strains in Africa

(Alizon et al., 1986; Benn et al., 1985; Magasiny et al., 1986; Desai et al., 1986). HIV-2 was originally located in West-Africa, where isolated viruses showed genetic polymorphisms (Clavel et al., 1986).

A characteristic feature of HIV-1 in cell cultures is the formation of syncytia. This phenomenon is dependent on cell surface expression of the differentiation antigen CD4 (Barre-Sinoussi et al., 1983; Lifson et al. 1986). The major targets of HI-virus are CD4+ cells (McDougal et al. 1985). The viral outer envelope glycoprotein gp110/120 has been shown to bind to the T4 (CD4) molecule (Dalgleish et al. 1984, McDougal et al. 1986). The T4 gene encodes the virus receptor and is expressed in the immune system and in the brain (Klatzmann et al. 1984, Maddon et al. 1986).

1.4.2. Serology of HIV infections

During HIV-1 infection, antibodies are produced to several viral proteins. According to western blot analysis antibodies are raised to gag proteins p17 and p24, to reverse transcriptase p66/p51 and to env proteins gp41 and gp120 (Lundberg 1988) (see Fig. 5). In most individuals infection is usually identified by detection of specific serum antibody to the virus, but in some patients the virus can be detected by virus isolation before the appearance of antibody or detection of antigen p24 (Sydow et al. 1988). Antigenemia could be detected in two weeks after infection and it persisted for 3-5 weeks (Allain et al. 1986). In most people with known HIV exposure and who have been followed prospectively, detectable antibody has developed within 3 months (Esteban et al. 1985). Horsburg et al. (1989) estimate that for the average individual the first detection of HIV antibody can be made at 2.4 months (SE 2.1).

The seropositivity is usually defined by the presence of at least anti-p24, anti-gp41 and anti-gp120 antibodies (Robey et al. 1985 Allan et al. 1985). Antibodies to the envelope proteins gp120 ans gp41 arise very early in the infection (Allain et al. 1986, Orgad et al. 1987)(Fig. 6). The level of anti-gp41 remains

high long after infection and persists into and throughout the AIDS phase when the anti-p24 levels may be dramatically reduced (Schupbach et al. 1985).

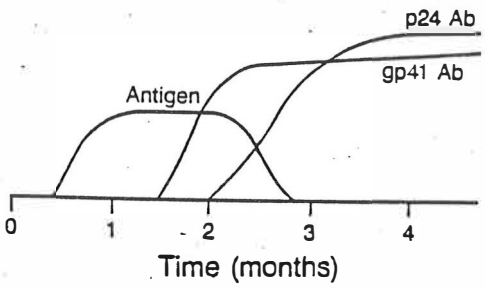

Figure 6. Time course for occurrence of specific markers following HIV-infection. The graph represents a composite of serial samples from 3 post-transfusion cases of HIV infection and 40 haemophiliac seroconverters (Allain et al. 1986).

1.4.3. Immunoreactive synthetic peptides of gp41

In the studies of the antigenic structure of HIV-1 with synthetic peptides several groups have located antigenic epitopes from one immunodominant antigenic region in the amino terminal part of gp41. This region, amino acids 578-613, seems to include several antigenic epitopes which are reactive with sera from individuals infected with HIV-1. In figure 7 there are known reactive peptides from this region and preliminary sensitivities with different HIV-1 positive serum panels.

1	ARILAVERYLKDQQLLGIWGCSGKLICTTAVPWNAS	
2	RILAVERYLKDQQLLGIWGCS	(360/373)
3	AVERYLKDQQLLGIWGCSGKLI	(319/322)
4	IWGCSGKLICTTAVPWNAS	('')
5	LGLWGCSGKLIC	(84/84)
6	ARILAVERYLKDQQLLGIWGCSGKLICTTAV	(34/38)

Figure 7. Synthetic peptides derived from the immunodominant region of gp41. The reactivity of each peptide was tested in indirect EIA. Number of positive sera in peptide EIA per confirmed HIV-1 positive sera are in parenthesis.
1. Entire sequence of the region. 2. Wang et al. 1986. Serum panel consist 228 AIDS and 145 ARC cases. Cases are diagnosed according to standard criteria of CDC. 3,4. Smith et al. 1987. Peptides 3 and 4 were used simultaneously in same EIA test. Results were compared to whole-virus EIA (HTLV III, Abbott Laboratories), false negatives were confirmed as positive in immunoblotting. 5. Gnann et al. 1987a. Positivity of the samples were classified as positive with commercially available whole-virus EIA. 6. Shoeman et al. 1987. Sera were classified as positive with Abbot and Electro Nucleonics HTLV III EIA kits and confirmed by immunoblotting.

Fine mapping of this region shows that the shortest reactive peptide is 7 amino acids, but for maximal reaction more than 10 amino acids are required. Figure 8 shows the location of the maximal antibody binding of two epitopes. In fig. 8A cysteine 609 seems to be important for the immunoreaction because its absence or susbstitution with serine eliminates the antibody reaction. In Fig. 8B (lines 8 and 9) threonine 611 could be deleted and cysteine 609 could be replaced with serine without any reduction in immunoreaction. In region 589-617 in the transmembrane protein gp41 there are at least two different antigenic determinants.

A amino acids 598-609

1	LGLWGCSGKLIC	22/22
2	GLWGCSGKLIC	21/22
3	LWGCSGKLIC	20/22
4	WGCSGKLIC	19/22
5	GCSGKLIC	14/22
6	CSGKLIC	21/44
7	SGKLIC	0/22
8	LWGCSGKL	0/22
9	LGLWGC	0/22
10	LGLWGCSGKLIS	2/22

B amino acids 589-617

1	DQQLLGIEGCSGKLICTTAVPWNASWSNK	
2	DQQLLGIEGC	0/15
3	LLGIWGCSGKLI	1/15
4	SGKLICTTAVPWNAS	15/15
5	SGKLICTTAVPWNASWSNK	15/15
6	CTTAVPWNASWSNK	4/15
7	AVPWNASWSNK	3/15
8	SGKLICT-AVPWNAS	15/15
9	SGKLISTTAVPWNAS	15/15
10	LLGIWGCSGKLI+CTTAVPWNAS	5/15

Figure 8. Fine mapping of two immunoreactive epitopes of gp41. The figures following the sequences indicate the proportion of reactive sera in serum panels.
A) Gnann et al. 1987b. Peptides were tested with 22 sera from patients from USA infected with HIV. Seropositivity was tested with commercially available whole-virus EIA (Litton). B) Huhtala et al. 1987. The peptides were tested with 15 sera from Finnish patients infected with HIV. Seropositivty was tested with two commercially available whole-virus EIA (Vironostika HTLV III, Organon Teknika and Labsystems HIV-EIA). In part B line 10 two separate peptides were used simultaneously.

1.4.4. Type specificity

Both in terms of the nucleotide sequences and serology HIV-2 is more close to simian immunodeficiency virus (SIV; STLV III_{mac}) than to HIV-1. The cross-reactivity between HIV-1 and HIV-2 is mainly limited to the viral core antigens, whereas envelope proteins are serologically quite distinct (Clavel et al. 1986, Brun-Vezinet et al. 1987).

Synthetic peptides have been used to define type-specific antigenic determinants of the envelope proteins of HI viruses. A series of synthetic peptides derived from different strains of HIV-1 and a peptide from the HIV-2 ROD were synthesized (Table 3) and tested with HIV-1 and HIV-2 positive human sera (Table 4). Cross-reactivities (2.5% and 6.1%) were observed between HIV-1 positive sera from the United States (2.5%) and Zaire (6.1%) and HIV-2 specific peptide (Gnann et al. 1987b).

Table 3. Sequences of synthetic peptides derived from transmembrane glycoproteins gp41 of HIV-1 and gp36 of HIV-2 (Gnann et al. 1987b). The amino acid sequences are published by Willey et al. 1986 (HIV-1 Z-3), Wain-Hobson et al. 1985 (HIV-1 LAVbru, ARV-2), Ratner et al. 1985 (HTLV IIIb,HTLV IIIRF), Alizon et al. 1986 (LAV-ELI, LAV-MAL) and Gyader et al. 1987 (HIV2-ROD).

Peptide number	Isolate name	Source	Amino acid sequence
1	HIV-1 (Z-3)	Zaire	LGLWGCSGKLIC
2	HIV-1 (LAVbru HTLV IIIB;WMJ-1 ARV-2, HTLV IIIrf)	France; USA	LGIWGCSGKLIC
3	HIV-1 (LAV-ELI)	Zaire	LGIWGCSGKHIC
4	HIV-1 (LAV-MAL)	Zaire	LGMWGCSGKHIC
5	HIV-2ROD	Cap Verde	LNSWGCAFRQVC

Table 4. Reactivity of different serum panels in peptide based EIAs (peptides 1-5). HIV-1 positive American sera from patients at different stages of HIV-1 infection were collected in San Diego, California and by CDC all over USA. Serum samples from Zaire were cultured for HIV and tested with commercial EIA (Litton). Selected Zairean samples were tested in HIV-1 immunoblotting. HIV-2 positive sera were from Guinea-Bissau and characterized by immunoblotting and immunofluorescence to HIV-1, HIV-2 and SIV antigens. The data shows the number of sera positive out of the number of sera tested and, in parenthesis the percentage of positive samples. ND = not done. The cut off for positivity was defined as the mean of absorbance values plus 3 standard deviations for a panel of 24 negative control sera.

	Peptide number				
Sera	1	2	3	4	5
HIV-1 infected, United States	162/163 (99.4)	40/40 (100)	39/40 (97.5)	35/40 (87.5)	1/40 (2.5)
Uninfected, United States	0/90	0/48	0/48	0/48	0/48
HIV-1 infected, Zaire	33/38 (86.8)	30/34 (88.2)	32/34 (94.1)	33/34 (97.1)	2/33 (6.1)
Uninfected, Zaire	0/21	0/20	0/20	0/20	0/20
HIV-2 infected, West-Africa	0/5	ND	ND	ND	5/5

In order to study the cross-reactivity and type-specificity of simian T-cell lymphotropic virus (STLV III) and HIV-1, synthetic peptides derived from the conserved region of the transmembrane protein of simian T-cell lymphotropic virus (STLV III) and corresponding peptides derived from HIV-1 gp41 (Fig. 9) were tested with two different groups of 20 sera. HIV-1 specific peptides detected all sera from the HIV-1 positive individuals from Sweden, but reacted poorly with sera from West Africa, whereas STLV-specific peptides reacted with all West African sera but detected only one serum in a panel from Sweden (Norrby et al. 1987; Fig. 10).

```
STLV III-1    ————————————————————————
STLV III-2                  ————————————————————————
STLV III      AIEKYLEDQAQLNAW-CAFRQVCHTT-VPWPNAS
              * * ** **  *  * *     * ** *** ***
HIV           AVERYLKDQQLLGIWGCSGKLIC TTAVPW NAS
HIV E34       ————————————————————————
HIV E32                     ————————————————————————
```

Figure 9. Alignment of the transmembrane region of STLV III (Kornfeld et al. 1987), and the corresponding region of HIV-1 (Ratner et al. 1985). Peptides derived from the regions are indicated with bars. Asterixes indicate conserved amino acids.

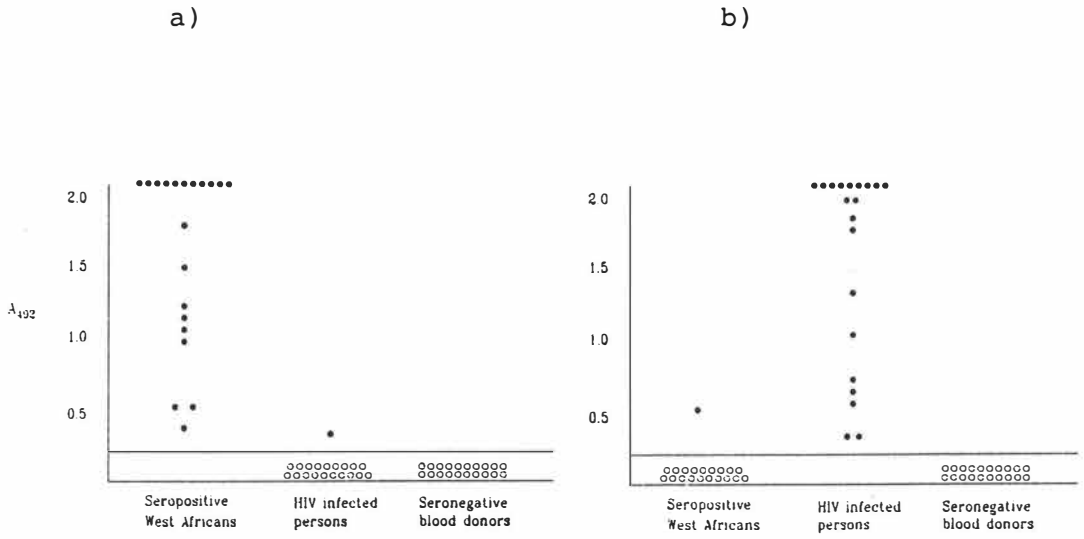

Figure 10. Reactivity of human serum samples (a) in STLV-III-peptide EIA (peptides STLV III-1 and STLV III-2) or (b) in HIV-peptide-EIA (peptides E32 and E34). A group (n=20) of sera from West-Africans with demonstrated antibodies to HIV-related viruses, a group (n=20) of sera from HIV-infected individuals and a group of sera (n=20) from blood donors with no demonstrated antibodies to HIV were tested in both assays. The peptide EIAs were performed as described by Smith et al. (1987).
The first group was previously tested by EIA with disrupted HTLV-IV virion as antigen and with presumed transmembrane protein gp32 of HTLV-IV in Western blot analysis. The second group, HIV-positive asymptomatic Swedish subjects, were positive in HIV-1 Western blot analysis. The third group, Swedish blood donors, were negative in commercial HIV and HTLV IV EIAs (Organon and Wellcome anti-HIV EIAs and HTLV-IV virion EIA) . The cutoff value was defined to be the mean of absorbance values of negative sera plus six standard deviations (Norrby et al. 1987).

1.5. Laminin

Laminin is a large and complex glycoprotein present as a major component of all basement membranes (Timpl et al. 1979, Chung et al. 1979). The molecular weight of the native protein is 850 000 and it was first indentified in the form of its polypeptide chains with Mr 200 000 (B-chains) and 440 000 (A chains) (Chung et al. 1979; Cooper et al. 1981). Later studies revealed two different B-chains, B1 (Mw 230 000) and B2 (Mw 220 000) both unrelated to A-chains (Cooper et al. 1981, Howe & Dietzcold 1983, Cooper & MacQueen, 1983). According to rotary shadowing electron microscopy and physical measurements, all chains are connected by disulfide bonds to form an asymmetric cross. Globules are observed in the middle and at the end of each short arm, and a large globule is observed at the end of the long arm (Engel et al. 1981). The long arm of the molecule is formed from alpha-helical segments, which are assembled in coiled-coil structures (Paulson et al. 1985). Computer analysis of the amino acid sequence of the B1 chain reveals the presence of structurally distinct domains that contain cysteine-rich repeats, globular regions and helical structures (Pikkarainen et al. 1987). The B2 chain has a similar multidomain structure to that in the B1 chain (Sasaki & Yamada 1987).

The laminin molecule interacts with other basement mebrane proteins, such as collagen IV and heparan sulfate proteoglycan. Studies using electron microscopy with laminin fragments and antibodies of known specificity have localized the collagen binding activity of laminin to the globular domains of the short and long arms (Rao et al. 1982; Laurie et al. 1986; Charonis et al. 1985, 1988). Heparan sulphate proteoglycan binds to laminin via the heparin binding domain at the end of long arm (Sakashita et al. 1980, Ott et al. 1982).

Biological studies suggest that laminin or laminin-like molecules may play a role in brain development (Liesi 1985, Letourneau et al. 1988, Liesi & Silver 1988) and in promoting neurite outgrowth (Liesi et al. 1984, Liesi 1985). Monoclonal

antibodies to proteolytic fragments have been used to localize the neurite outgrowth-promoting domain near or in the heparin-binding region of the laminin molecule (Edgar et al. 1984, Engvall et al. 1986, Edgar et al. 1988) and the corresponding proteolytic fragments have been shown to promote attachment of chicken peripheral neurons (Edgar et al. 1984, 1988).

A synthetic peptide from the cysteine-rich region of the B1 chain short arm was found to be active in cell attachment, receptor binding, and migration. This pentapeptide blocked laminin mediated cell adhesion and migration and was able to prevent the metastasis of tumor cells in experimental animals (Graf et al. 1987a, Graf et al. 1987b, Iwamoto et al. 1987, Iwamoto et al. 1988). Another peptide from the adjacent globular domain of the B1 chain has been reported to be active in cell attachment and heparin binding (Charonis et al. 1988). A synthetic pentapeptide from above the carboxyl globule on the long arm of the A-chain was found to promote cell adhesion, spreading, migration and neurite outgrowth. In addition this peptide was able to stimulate axonal-like progress of cerebellar neurons and PC-12 cells (Tashiro et al. 1989, Sephel et al. 1989). A schematic representation of the laminin molecule is shown on page 73 (Fig. 32).

2. Aims of the present studies

Labsystems Research Laboratories, together with the Finnish Academy started a project in 1984 to create the first laboratory for synthetic peptides in Finland. The purpose of this project was to bring solid phase peptide synthesis technology to Finland, as well as to study biological functions of synthetic peptides and anti-peptide antibodies. The investigations presented in this thesis originate from that main project. The aims of the studies were as follows:

1) To purify the human endogenous retrovirus gene product from human cultured choriocarcinoma cells. The purification steps were monitored with antibodies to a synthetic peptide derived from the sequence of human endogenous cDNA.

2) To apply computer analysis in order to study the antigenic structures of viral proteins, as well as secondary structures of structural proteins.

3) To use synthetic peptides in serological studies to characterize continuous antigenic determinants in Human Immunodeficiency Virus, and to detect the human antibodies, which have been raised to viral proteins during infection.

4) To use synthetic peptides in locating the cell attachment and neurite outgrowth promoting domains in the laminin molecule.

3. Materials and methods

3.1. Principles of solid phase peptide synthesis (SPPS)

The method of solid phase peptide synthesis was first described by Bruce Merrifield (Merrifield 1963). In this procedure the synthesis occurs on the surface of an insoluble polymer. The growing peptide is attached on the polymer while uncoupled amino acids and coupling reagents are dissolved in organic solvents and are easily removed by filtering. Insoluble polymer can be easily washed and deprotected before the next synthesis cycle.

3.1.1. Solid phase support

Copolymerization of styrene with 1% divinylbenzene added to secure insolubility through crosslinking, is still the basic method in development of new polymers (Fig. 11). These gels become highly solvated and swollen in organic solvents of intermediary polarity such as dichloromethane, dimethylformamide, and aromatics (Merrifield 1984). According to the original Merrifield procedure the resin is converted to a chloromethyl derivate. This group is allowed to react with the carboxyl group of a protected amino acid. The reaction forms a benzyl ester linkage between the first amino acid and the polymer support.

After anchoring the first amino acid on the resin the amino terminal protecting group can be removed and the second amino acid can be coupled. After completing the peptide synthesis the peptide is removed from the resin. Strongly acid reagents are required for benzyl ester cleavage. Commonly used strong acids are hydrogen fluoride and trimethylsulfonylfluoride acid.

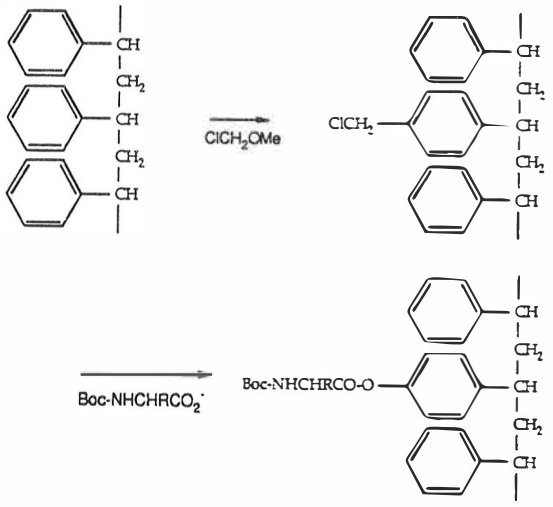

Figure 11. Chloromethylation of the polystyrene and reaction of chloromethyl derivate with N-protected amino acid (Atherton & Sheppard 1989)

3.1.2. Formation of the peptide bond

The most widely applied approach to form a peptide bond involves the use of "coupling reagents" such as dicyclohexylcarbodiimide (Fig. 12).

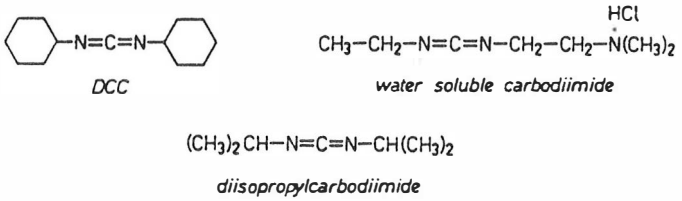

Figure 12. Dicyclohexylcarbodiimide, watersoluble carbodiimide and diisopropylcarbodiimide (Bodanszky 1984)

There are three possible pathways to form peptide bonds by using carbodiimides as coupling reagents (Fig 13).

A) The free carboxyl group reacts with DCC producing o-acyl-isourea intermediate. Reaction of the o-acylisourea with un-reacted carboxyl component yelds a symmetrical anhydride that reacts with free amino group. The by-product is N,N'-dicyclo-hexylurea.

B) Asparagine, glutamine and arginine are very unstable as their corresponding anhydride. These amino acids can be converted to the active esters with hydroxybenzotriazole (HOBt).
C) Carbodiimides can be added to the mixture of the carboxyl and amino components. The by-product of this reaction is N,N'-dicyclohexylurea in DCC mediated coupling.

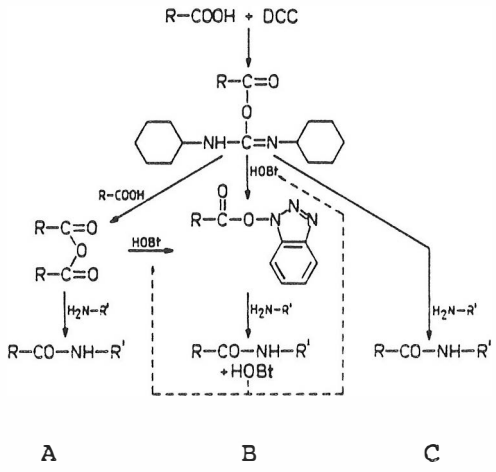

Figure 13. Three pathways for peptide bond formation (Bodanszky 1984).

3.1.3. Protection of the alpha amino group

To avoid the formation of polymers the alpha amino group of the amino acid is protected. The most widely used protection group is tert-butyloxycarbonyl (t-Boc)(Fig. 14). The bond between the amino group and t-Boc group is stable in weak acids but can be removed with strong acid such as Trifluoroacetic acid.

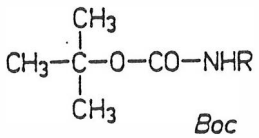

Figure 14. Tert-butyloxycarbonyl group (t-Boc)(Bodanszky 1984).

3.1.4. Protection of side chains

To avoid reactions between amino acid side chains and free amino groups some of the side chains are protected. The choice of benzyl ester groups for side chain protection also allows the cleavage of these groups simultaneously with the cleavage of the peptide from the resin. The reactivity can be adjusted equally for all groups by appropriate nuclear substitution of the benzylic ring (Table 5).

Table 5. Commonly used side chain protection groups in t-Boc based peptide synthesis (Bodanszky, 1984, Atherton & Sheppard 1989).

AMINO ACID	AMINO ACID DERIVATES	
Arginine	p-Toluenesulfonyl	(Tos)
Aspartic Acid	Benzyloxy	(OBzl)
Cysteine	4-Methylbenzyl	(4-Me-Bzl)
Glutamic Acid	Benzyloxy	(OBzl)
Histidine	p-Toluenesulfonyl	(Tos)
Lysine	Clorobenzylcarbonyl	(Cl-Z)
Serine	Benzyl	(Bzl)
Threonine	Benzyl	(Bzl)
Tryptophane	Mesitylene-2-sulfonyl	(Mts)
Tyrosine	2-Bromobenzylcarbonyl	(2-Br-Z)

3.2. Peptide synthesis

Peptides were synthesized by using the solid phase method and a Vega Coupler (250C). For the synthesis Boc-Cys(MBzl)-O-resin was used as the solid phase support (Peninsula Laboratories, Belmont CA) and alpha-amino t-butyloxycarbonyl protected amino acids were used (Sigma, St. Louis, MO) during synthesis. Dicyclohexylcarbodiimide (Sigma) was used as the coupling reagent. The synthesis procedure was as follows (Fig.15):

Synthesis was started with 5 grams of the resin precoupled with 0.5 mmol of Boc-Cys(MBzl) via ester lingage. The first t-Boc group was removed with 40% trifluoroacetic acid in dichloro-methane (DCM) for 30 min. The deblocking was confirmed with the Kaiser test (Kaiser et al., 1970). A small amount of the resin was taken in a test tube and equal volumes (50 µl) of 1 mM po-tassium cyanide (Sigma) in pyridine (Merck), Phenol (Merck) 4g/ml in ethanol and ninhydrin (Merck) 50 mg/ml in ethanol. The mixture was incubated at 100° C in a water bath for 10 min. The reaction was determined as positive or negative by eye. If the reaction mixture turned blue it was considered positive.

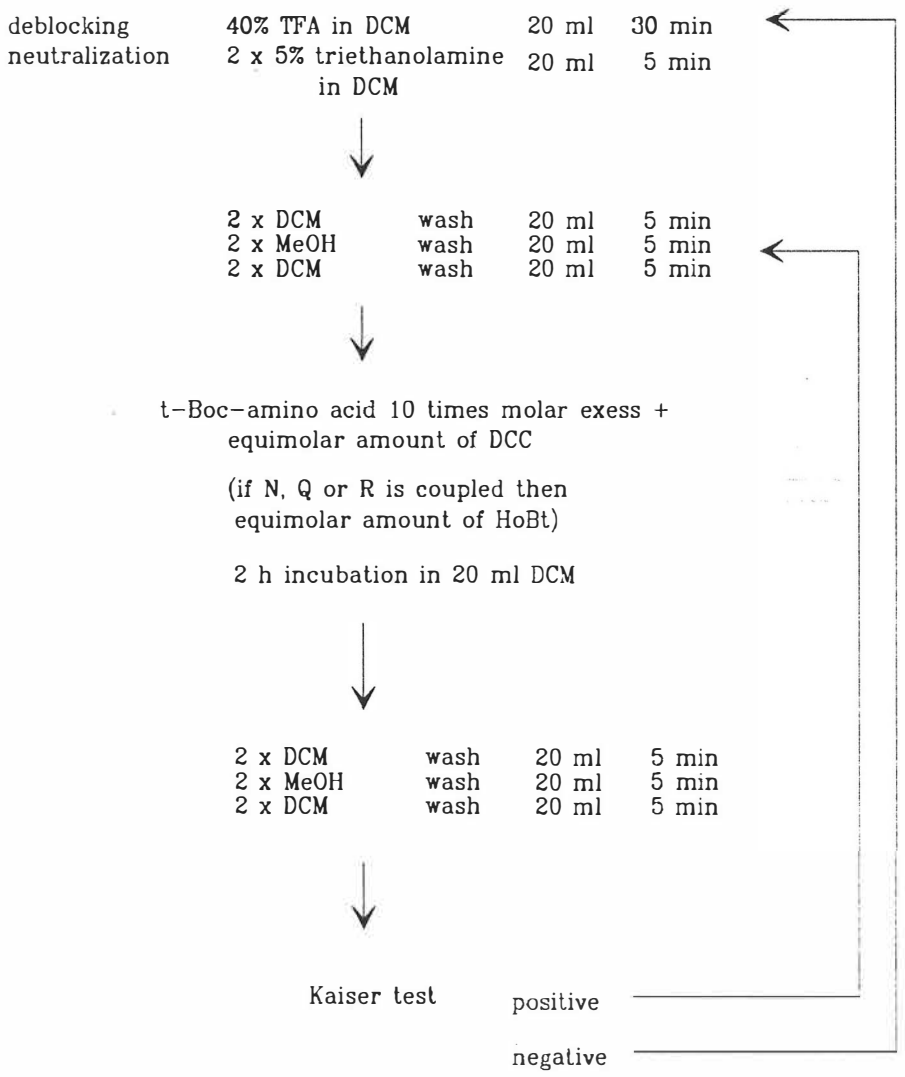

Figure 15. Synthesis cycle in t-Boc based peptide synthesis using DCC as coupling reagent.

After obtaining a positive Kaiser test, t-Boc protected amino acid with an equimolar amount of DCC was coupled to the free amino terminus. The molar concentrations of the protected amino acid and DCC were 10 times that of the first amino acid on the resin. In the case of N, Q or R an equimolar amount of 1-hydroxybenzotrazole (HoBt, Sigma) was added.After each cycle the coupling of the amino acid was tested with the Kaiser test as described above. After the Kaiser test was considered as negative, the t-boc group was removed and the next t-Boc protected amino acid was added.

After the synthesis was completed the peptide was removed from the resin and the side-chains were removed from the amino acids with hydrogen fluoride using a cleavage machine (Peninsula). The resin was collected to the reaction chamber with anisole (Merck), for protection, to a final concentration of 5%. 10 ml of HF gas (AGA, Helsinki, Finland) was then condensed in the cleavage apparatus, while the reaction chamber was cooled in a methanol bath with dry ice. After condensation the resin was mixed in an ice bath for 30 min. HF was removed by evaporation with nitrogen gas until the resin was dry. Free peptide was dissolved in the HPLC starting buffer (0.1% TFA in H_2O) and sintered from the resin.

Cleaved peptides were purified with reverse-phase high pressure liquid chromathography [Vydack C18 reverse phase column (Vydack); Kontron HPLC apparatus)]. Peptides were absorbed to the column with 1% TFA in H_2O as running buffer and eluted with a linear 60 min gradient, using 60% acetonitrile as eluting buffer. The flow rate was 6 ml/min and 3 ml fractions were collected. The peptides were monitored at 220 nm. Fractions were tested with the Kaiser test and analytical HPLC. Positive fractions were pooled and lyophilized.

The amino acid sequences were confirmed by automated Edman degradation with a gas-phase sequencer (Model 470A, Applied Biosystems). The purity of the peptides were approximately 99% based on amino acid sequence data (Table 6).

Table 6. Peptides synthesized for this study. Sequences A15-P3 are published by Ratner et al. 1985, P4 by Kornblihtt et al. 1985, P5 by Pierschbacher & Ruoslahti 1984, P7, P20 and P32 by Barlow et al. 1984 and P31 by Graf et al. 1987. Peptide P6 is a hydrophilic nonsense peptide. Amino acids in parenthesis are additional to facilitate the coupling of the peptides to the carrier protein. Amino acid numbering is according to Swiss-Prot protein sequence data base.

A15	SGKLICTTAPWNAS(C)	HIV-1 gp41	599-613
B18	LIEESQNQQEKNEQELLE(C)	''	645-662
P1	DQQLLGGW(GC)	''	589-596
P2	DEPEGIEEEGGERDDPS(GC)	''	668-684
P3	SLIEESQNQQEKNEQELLE(GC)	''	644-662
P4	ESKPEAEET(C)	Fibronectin	57-65
P5	GRGDSP	''	1492-1497
P6	DYQKLNNAFGC	nonsense peptide	
P7	KAKDEMKASD	laminin	152-161
P20	RNIAEIIKDI(GC)	''	185-194
P31	CDPGYIGSR	''	994-1002
P32	KQEAAIMDYNRNIAEIIKDIHN LEDIKKTL	''	175-204

3.3. Computer analyses

3.3.1. Hydrophobicity

Hydrophobicity was calculated using Eisenbergs normalized consensus indices (Eisenberg, 1984b), using the program designed by Dr. Joszef Soós, Institute of biophysics, Hungarian Academy of Science, Szeged, Hungary, and an Olivetti M24 microcomputer (Olivetti, Torino, Italy). Hydrophobicity was averaged using seven amino acid window and assigning the average hydrophobicity value to the fourth amino acid.

3.3.2. Hydrophobic moment plot of laminin B2 chain

The hydrophobic moment plot was calculated according to the algorithm of Eisenberg et al. (1984a) using the program written by Dr. Joszef Soós and an Olivetti M24 microcomputer (Olivetti). The numerical hydrophobicity of an individual amino acid was from Eisenberg's normalized consensus index (Eisenberg, 1984a)

and the window for averaging hydrophobicity was 11. The hydrophobic moment plot was calculated using a 100 degree angle for the proposed alpha-helical structure of the laminin B2 chain in an 11 amino acid window. The μ-value of 11 amino acid window was given to the sixth amino acid.

3.4. Cell cultures

3.4.1. Cell lines

3.4.1.1. Human choriocarcinoma cells

For purification of Mr 75000 polypeptide, human choriocarcinoma JEG-3 cells (A.T.C.C. HTB 36; American Type Culture Collection, Rockville, MD), which are known to secrete human chorionic gonadotropin, were used. Cells were grown in roller bottles (1350 cm^2) to confluency or on Petri dishes as monolayer cultures to confluency in Eagle's minimal essential medium (MEM) with 10% (v/v) fetal bovine serum. 1-2% of the cells had a syncytiotrophoblastic morphology and the rest of the cells were cytotrophoblast-like.

3.4.1.2. Transformed T-cells

Transformed T-cells (C10/MT2 and H9) were cultured in continuous suspension culture in RPMI 1640 culture medium containing 10% FBS, penicillin and streptomycin.

3.4.2. Organotypic cultures

For neurite outgrowth assays, newborn mouse spinal cord and cerebellum, or 16-day-old mouse embryo dorsal root ganglia tissues were used. Tissue pieces were grown in Trowell-type organotypic cultures on nucleopore filters (pore size 1 μm; Plesanton, CA) placed on a metal grid in a serum free, chemically defined NI-medium (Selak et al. 1985).

3.5. HIV-1 virus

3.5.1. Infection

In all studies HIV-1 strain HTLVIIIb was used (Popovic et al. 1984). The virus strain was received in H9 cell culture. For immunofluorescence and radioimmunoprecipitation assays C10/MT2 and H9 cells were infected with HIV-1 virus. Cells were treated with polybrene (2 µg/ml medium) for 20 h at 37° C. HIV-1 virus was centrifuged from the cell-free medium of virus producing culture and resuspended to 1ml of culture medium. Before infection polybrene treated cells were washed once with culture medium and suspended to medium $5x10^6$ cells/ ml. One ml of cell suspension and 1 ml of virus suspension were mixed and incubated for 2 hours at 37° C. After infection cells were washed and cultured in roller bottles in culture medium containing 10% FBS, penicillin and streptomycin.

3.5.2. Purification

For immunoblotting HTLVIIIb was purified from the supernatant of virus producing cell line H9. Virus was first consentrated with Pellicon apparatus and the final purification was done by sucrose density gradient ultracentrifugation. Virus-particles were distrupted with Laemmli's (1970) sample buffer containing 1% beta-mercaptoethanol by heating 3 min at +100° C.

3.6. Biochemical methods

3.6.1. Triton X-114 two-phase separation

Triton X-114 (Sigma) was precondensed and used at a 1% (v/v) concentration in TEN buffer. JEG-3 cells, grown on Petri dishes, were washed three times with phosphate buffered saline (PBS; 10 mM phosphate/150 mM NaCl, pH 7.4). The dishes were cooled on ice and 1 ml of ice-cold 1% Triton X-114 was added. The dishes were tilted gently for 10 min and the material was collected into a Minifuge tube. Insoluble material was sedimented and the super-

natant was phase-separated as described by Bordier (1981). All three fractions, insoluble material (collected in 50 µl), detergent phase (50 µl) and water phase (950 µl), were analysed by immunoblotting with anti-Sp-23 as antibody.

3.6.2. Gel filtration

For the first purification step of Mr 75000 polypeptide, JEG-3 cells, cultured in roller bottles, were washed three times with PBS and harvested with a rubber policeman by using 1% (w/v) Triton X-114 in TEN buffer (50 mM Tris/HCl / 10 mM EDTA / 150 mM-NaCl; pH 7.4). Insoluble material was removed by centrifugation at 10 000 g for 15 min. at +4 C. Hydrophilic fraction was run in a TSK 3000 gel filtration column (21.5 mm x 300 mm) (Toyo-Soda, Tokyo, Japan) and HPLC (Varian 5000 liquid cromatograph, Varian UV-100 detector and Varian 4270 integrator). The running buffer was 50 mM-Tris/HCl 150 mM NaCl, pH 6.5, the flow rate 3 ml/min and the run was monitored at 280 nm. Fractions (3 ml each) were collected and tested by the immunoblotting technique with anti-Sp23 antibody. Positive fractions were pooled, freeze-dried and dissolved in 1/6 vol. of distilled water. The solvent was changed to 20 mM Tris/HCl, pH 7.5, and a Pharmacia PD 10 gel filtration column was used. A single 3 ml fraction was collected.

3.6.3. Anion-exchange chromatography

After gel fitration, anti-sp23 positive fraction pool was run in anion exchange chromatography using a Pharmacia Mono Q column and a Pharmacia FPLC apparatus (GP-250 gradient programmer, P-500 pumps and UV-1 single path monitor) and monitored at 280 mm. The running buffer was 20 mM Tris/HCl, pH 7.5 (buffer A). The column was first eluated with 10% (v/v) buffer B (20 mM tris/HCl, 560 mM-NaCl, pH 7.5), and then the proteins were eluted with a linear 10-100% buffer-B gradient for 36 min at a flow rate of 1 ml/min. Fractions (1ml) were collected and tested as described above.

3.6.4. Reverse-phase chromatography

In the last step of the purification procedure, the preparation from anion exchange was run with the pharmacia Pro-RPC reverse-phase C-18 column with the Varian HPLC system. The running buffer was 0.1% TFA in distilled water (buffer C) and bound proteins were eluted with acetonitrile containing 0.08% TFA (buffer D) The program was: 0-7 min, 0% D; 7-10 min, 30% D; 10-20 min, 50% D; 20-23 min, 100% D;23-27 min, 100% D; and 27-30 min 0% D. The flow rate was 0.7 ml/min and the wavelength 218 nm. Protein-containing peaks were collected manually and tested as described above.

3.6.5. Peptide-BSA conjugates

For facilitating the attachment of the peptides on the micro-titration plates and on the glass coverslips peptides A15 and B18, derived from gp41 of HIV-1, and peptides p20 and p31, derived from laminin (see page 36), were coupled to bovine serum albumin (BSA) using m-Maleimidobenzoic acid N-Hydroxysuccinimide ester (MBS) (Liu et al. 1979).

BSA was first activated with DMF. 20 mg of BSA (A-7030, Sigma) dissolved in 1 ml of PBS, was mixed with 600 µg MBS (M-8759) dissolved in DMF, and stirred at room temperature for 30 min. Free MBS was removed with a Sepharose G15 (Pharmacia) column, using PBS pH 6.4 as running buffer. Activated BSA was then mixed with the peptide, diluted in PBS in a molar ratio of 1:15, and stirred at room temperature for 4 hours.The peptide-BSA conjugate was dialysed against PBS over night at room temperature to remove free peptide (Spectra/Por molecularporous mebrane tubing, mw cut off 12-14000, Thomas Scientific, USA). The coupling efficiency was measured by sequencing (see peptide synthesis).

3.6.6. Activation and coating of glass coverslips

The glass coverslips were acivated with 3-aminopropyltrimethoxy-silane followed by treatment with glutaraldehyde (Aplin & Huges

1981). After treatment, purified mouse laminin (100 µg/ml, 100 nM; Timpl et al. 1979) or BSA-conjugated peptides p20 and p31 alone or in combinations (100 ng/ml, 100 nM) were covalently coupled on the cover slips. The concentration of the peptides were determined as described above.

3.7. Immunochemical methods

3.7.1. Affinity chromatography

For purification of human antibodies to the highly reactive epitope of HIV-1 gp41, the synthetic peptide A15 was coupled to AH-Sepharose-4B (Pharmacia) via the carboxy terminal cysteine residue, using MBS (Sigma) as coupling reagent. AH-Sepharose-4B (100 mg) was first swollen in potassium phosphatebuffer pH 7.4 containing 150 mM NaCl. MBS solution (3% in dimethylformamide, 50 ul, Sigma) was added to 1 ml AH-Sepharose-4B suspension, which was then stirred gently at room temperature for 30 min. The AH-Sepharose suspension was washed twice with PBS and 1 mg of the peptide was added. The suspension was stirred for 4 hours at room temperature. The peptide-AH-Sepharose-4B was mixed with 2 ml Seharose-4B (Pharmacia) and used for preparation of the affinity column. A pool (1.5 ml) of 10 HIV-1 antibody positive sera, all of which were highly immunoreactive in the peptide EIA (see below), was passed through the affinity column. After the elution of unbound sera from the column with PBS, it was washed with 500 mM NaCl in 10 mM phosphate buffer (pH 7.4). Column bound Ig molecules were eluted with 2 ml of 100 mM Glycine-HCl buffer (pH 2.8) monitoring at 280 mm (UV-1 single path monitor, Pharmacia). The acid buffer was changed to PBS using a Sephadex G25 (Pharmacia) column.

3.7.2. Polyacrylamide gel electrophoresis and immunoblotting

For monitoring the purification steps of Mr 75000 polypeptide, portions of the fractions (50 ul) were mixed with 1 vol. of Laemmli's (1970) sample buffer and the proteins were separated by SDS/polyacrylamide gel electrophoresis, with 10% (w/v) poly-

acrylamide slab gels under reducing conditions. Coomassie Brilliant Blue (0.2% (v/v)) was used to stain proteins in the gels. The gels were destained with 10% (v/v) acetic acid.
For immunoblotting, the proteins were transferred electrophoretically from the gel to nitrocellulose sheets and immunoblotted. Proteins were immunostained with rabbit anti-Sp23 antiserum at 1:500 dilution in TEN-Tx buffer (50 mM Tris/HCl (pH 7.0)/5 mM EDTA/150 mM-NaCl/ 0.05% Triton X-100). The immunoreactive polypeptide band was detected with peroxidase-conjugated anti-rabbit IgG (DAKO, Copenhagen, Denmark). The pre-immune serum of the rabbit was used as a control (Suni et al., 1984).

The reactivity of human sera and affinity-purified Ig fractions were characterized by immunoblotting. HIV-1 polypeptides were separated from purified virus (4 µg) using 10% (w/v) polyacrylamide gels under reducing conditions (1% beta-mercaptoethanol). Peptide A15 (3µg) conjugated to BSA via MBS was also run in SDS-PAGE under the same conditions. After separation, proteins were transferred to nitrocellulose and tested for immunoreactivity with HIV-1-positive or HIV-1 negative sera, diluted 1:100, or with purified anti-peptide Ig-fraction diluted 1:5 (5 µg/ml); these dilutions gave equal absorbance values in peptide A15 EIA. Also, peptide A15-BSA conjugate was tested for its immunoreactivity with rabbit anti-BSA antiserum (Cappel Laboratories, Cochranville, PA). Bound antibodies were detected by rabbit anti-human IgG-peroxidase conjugate (DAKO) and by swine anti-rabbit Ig-peroxidase conjugate.

3.7.3. Radioimmunoprecipitation assay (RIPA)

For preparation of RIPA antigen log-phase cultures of HIV-1-infected C10/MT2 cells were starved in cysteine-free RPMI culture medium for 30 min. After starvation 2 x 10^6 cells were metabolically labelled with ^{35}S-cysteine (0.5 mCi) in suspension at +37° C for 22 h. The cell lysate was prepared by pelleting the cells and washing them in PBS and using a lysis buffer containing non-ionic detergent (0.5 ml; 10 mM Tris-HCl containing 140 mM $MgCl_2$, 1 mM dithiotreitol, 1 mM PMSF and 0.5% NP-40, pH 8.0). For the precipitation assay, 30 µl antigen preparation was

used per 15 µl serum. The precipitates were collected with protein A Sepharose (Pharmacia) and, after extensive washing, were boiled with SDS-beta-mercaptoethanol (1%) and analysed in SDS-PAGE (12%), followed by autoradiography.

3.7.4. Immunofluorescence

HIV-1 infected H9 cells were attached to glass slides coated with poly-L-lysine (Sigma). The cells were fixed with 3.5% paraformaldehyde (Riedel-de-Haen, Hannover, FGR) in PBS. The fixed cells were permeabilized with 0.05% saponin (Merck) in PBS and treated with rabbit serum in PBS-saponin. Cells were stained with the unfractionated HIV-1 serum pool or with an HIV-1-negative human serum diluted 1:50 or with anti-peptide Ig-fraction (23 µg/ml) for 30 min at 37°C . The cells were washed three times with PBS-saponin and the bound antibodies were detected by incubating for 30 min at 37°C with biotinylated sheep anti-human IgG with fluorescein-streptavidin conjugate (Amersham, Amersham, UK).

3.7.5. Enzyme immunoassays

For enzyme immunoassays (EIA), peptide-conjugates A15-BSA and B18-BSA were used as antigens. Peptide conjugate, diluted in PBS, was added to each well of a polystyrene microtitration plate and incubated overnight at room temperature. Each serum sample was tested as duplicates. Serum specimens were diluted 1:40 with 1% BSA in PBS and 0.02% Tween 20 and 100 µl of the sample was added to each well. The plates were incubated for 1h or 2h at 37 C and the unbound antibodies were removed by three washes (200 l) with PBS 0.02% Tween 20. The plates were then reacted for 1 h at 37 C with swine anti-human IgG conjugated with alkaline phosphatase (Labsystems) followed by three washes as above and exposure to para-nitrophenyl phosphate (Sigma) as substrate. The absorbance values were measured at 405 nm using a Multiscan microtitration plate reader (Labsystems). The mean of the two absorbance values of each sample was calculated. In order to avoid the effect of variations between the different EIA

determinations the results were calculated according to the following equation and expressed as enzyme immunoassay units (EIUs):

$$\text{Value in EIUs} = \frac{[A(S) - A(B)]}{[A(C) - A(B)]} \times 100$$

where A(S) is the absorbance of the sample, A(B) is the absorbance of the reagent blank, and A(C) is the absorbance of the positive control.

The reactivity of the purified anti-peptide Ig fraction and unfractionated HIV-1 positive sera were characterized by a Labsystems whole-virus EIA kit and by the peptide EIA. The unfractionated HIV-1 antibody-positive sera, HIV-1 negative-sera and the purified anti-peptide Ig fraction were diluted from 1:5 to 1:640. The IgG concentration of the purified anti-peptide Ig fraction was determined by measuring the absorbance of the eluate at 280 nm (1% = 6.9). As a negative antigen control, peptide B-18-BSA conjugate was used.

3.8. Serum samples

Rabbit anti-sp23-peptide antibodies were a gift from Dr Stephen Oroszlan, Biological Carcinogenesis Program, National Cancer Institute, Frederick, MD (Suni et al. 1984).

Human sera were from 152 patients at various stages of HIV-1 infection from Finland (n = 138) and Africa (n = 14) and five sera were from patients infected with HIV-2. A panel of 144 sera were verified on initial testing to be positive both in whole-virus EIA and in immunoblotting (see below). Eight of the cases were known to be exposed to HIV-1 by sexual transmission and to be seroconverters, and serially collected sera were available. All of these cases were seronegative both in whole-virus HIV-1 EIA and immunoblotting on initial testing. Sera of blood donors (n = 1000) negative in whole-virus EIA served as control. In addition, patients (n = 90) with different clinical disorders commonly giving false-positive reactions in whole-virus EIA were

studied. This group included patients undergoing hemodialysis (n = 20), with kidney transplantation (n = 20), and patients with miscellaneous infections other than HIV-1 (n = 50). All serum specimens were stored at -20 C until assayed.

All human serum specimens were tested by Vironostica HTLV III (Organon, Teknika Oss, Holland) whole-virus EIA kit. This test is an indirect EIA using anti-human Ig-HRP as conjugate and purified T-lymphtropic virus subclass III as antigen. Serum specimens were diluted 1:100. Serum samples giving a positive reaction in whole-virus EIA were repeatedly tested in EIA, and the positive reactions were confirmed by immunoblotting using whole-virus antigen strips prepared either by DuPont (Boston, MA) or Labsystems. HIV-2 antibodies were assayed by Elavia Ac-Ab-Ak II kit (Diagnostics Pasteur, Paris, France).

3.9. Cell function tests

3.9.1. Survival and competition assays

The cells were mechanically dissociated from newborn rat (Wistar) cerebellum (Liesi et al. 1983) and grown on glass coverslips coated with laminin. To test the purity of the peptides and the additional effects of the peptide preparations, synthetic peptides P1-P7 were used as control peptides. In survival studies, the free peptides were applied at 100 µg/ml and in competition assays at concentrations of 1-5 µg/ml. In time-lapse-video recording experiments, 50 µg/ml of peptide p20 were added to cultures of neurons that had already extended neurites on laminin. Time-lapse-video recordings were peformed using National NV-8030 time-lapse recorder installed with an Olympus IMT inverted research microscope with high magnification oil immersion optics.

3.9.2. Neurite outgrowth and cell attachment assays

Organotypic cultures were tested with peptides p20, p31, p32, p6 or p7 (see page 36) dissolved in serum free NI-medium. All

peptides were used at 100 nM (0.1 µg/ml). In DGR cultures the medium also contained 10 nM of 7S-NGF (Collaborative Research, Lexington, MA). In some experiments, the cultures were grown in the presence of soluble native mouse laminin, also applied at 100 nM (100 µg/ml). After 24-48 h the cultures were fixed with cold (-20° C) methanol for 30 min, the tissue explant was scraped off and the filters were exposed to rabbit polyclonal antibodies to neurofilament proteins, diluted 1:100 in PBS, for 24 h at 4° C. The filters were rinsed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit immunoglobulins (Wellcome Research Laboratories Beckenham, UK) diluted 1:20 for 30 min at room temperature followed by rinsing with PBS (Liesi et al., 1983).

For cell attachment assays, coverslips were coated with the peptides p20-BSA and p31-BSA alone, or in the combinations indicated and with native laminin. Cells from newborn rat (Wistar) cerebellum were mechanically dissociated and plated on the coverslips at a density of 10^5/ml in the NI-medium. Quantitation of attached neurons and their neurite extensions was performed after 24 h, by counting all live attached neurons with long neurites (> 5 times cell soma) using a Leitz Labvert inverted microscope with phase contrast optics. In these experiments, the entire 18 mm coverslip was screened, and all single live neurons counted. With each substrate, 4-5 coversplips were screened. The arithmetical mean of attached neurons with neurites on laminin substrate in one experiment was considered to be 100%, results from 6 separate experiments were used for calculations.

4. Results and discussion

4.1. Purification of M_r 75 000 protein from cultured human choriocarcinoma cells (I)

The purification steps of M_r 75000 polypeptide from cultured JEG-3 cells were monitored with rabbit anti-sp23 antibodies in SDS/polyacrylamide gel electrophoresis and immunoblotting.
First the solubility of the protein was determined by the Triton X-114 two phase system. As shown in figure 15a, all of the M_r 75 000 polypeptide separated totally into the hydrophilic phase. On the basis of this result, a phase-separation method was used as the first purification step. For the purification of Mr 75000 protein, the JEG-3 cells were grown in roller bottles.

After phase-separation, soluble proteins were first gel filtered with an HPLC gel permeation column. The M_r 75000 reactivity migrated as a single broad peak in fractions 17-24, and was separated from small-sized proteins (Fig. 15b). The Triton X-114 detergent molecules absorb at 260 nm (Tiller et al., 1984) causing a large peak in the front of chromatogram. Albumin (M_r 67000) migrated in fractions 20-24, indicating that the M_r 75000 component (fractions 17-24) was a monomeric protein.

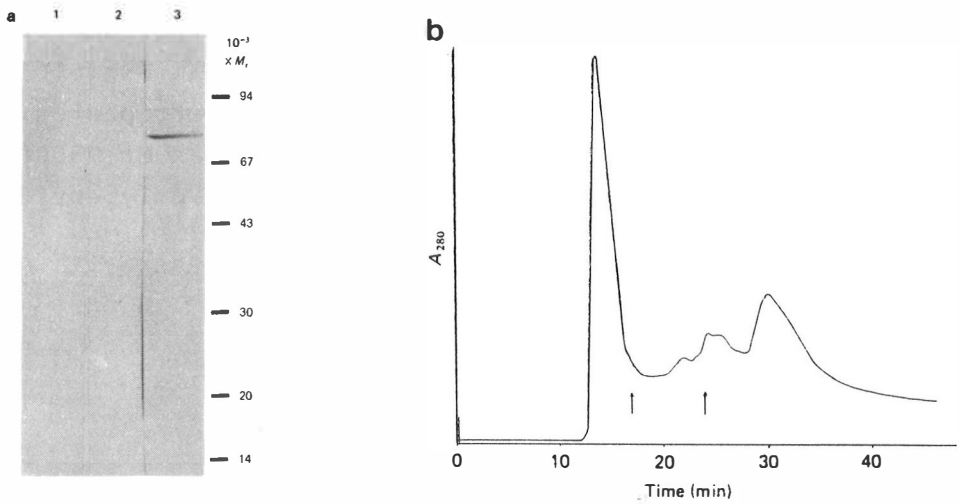

Figure 15. a) The fractions of Triton X-114 phase separation of cultured choriocarcinoma cells were analysed by SDS/polyacrylamide gel electrophoresis followed by immunoblotting with anti-sp23 as antibody. Insoluble material (lane 1), the lower phase (hydrophobic; lane 2) and the upper hydrophilic phase (lane 3). Anti-sp23 reactivity can be seen only in lane 3.
b) Gel filtration HPLC (TSK 3000) chromatogram of Triton X-114-extracted choriocarcionoma cells. The arrows indicate the fractions which were positive in immunoblotting with anti-sp23 antibodies.

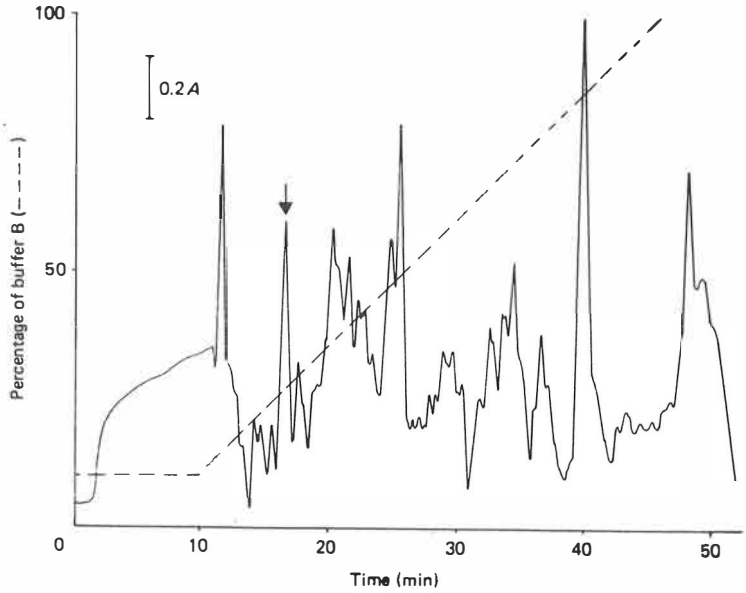

Figure 16. Anion-exchange HPLC (Mono Q) chromatographic run of the pooled positive gel-filtration fractions. Buffer A = 20 mM Tris-HCl, pH 7.5. Buffer B = 20 mM Tris/HCl, 560 mM NaCl, pH 7.5. The elution position of immuno-reactive material is indicated by the arrow.

The anti-sp23 positive fraction pool from gel filtration was run in anion exchange HPLC. The positive fractions were eluted as a single peak at 25% buffer B (150mM NaCl)(Fig. 16). As seen in figure 17 the SDS/polyacrylamide-gel electrophoresis showed that the major protein in the active peak was Mr 75000 protein. The major contaminants at this stage were polypeptides of M_r 30000, M_r 50000 and M_r 70000.

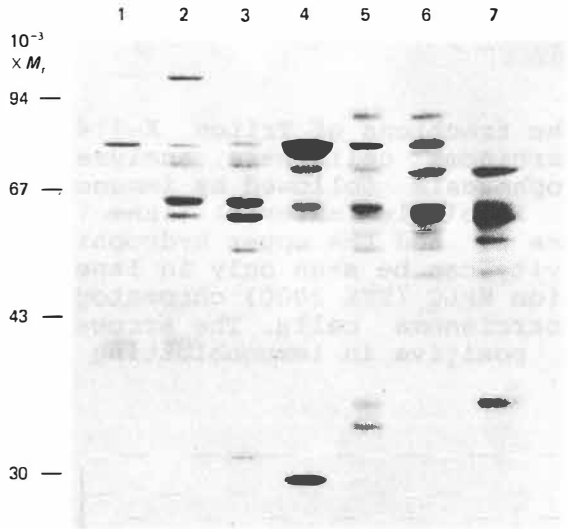

Figure 17. SDS/polyacrylamide gel electrophoresis of fractions 14-20 collected from anion exchange chromathography followed by Coomassie Brilliant Blue staining.

In the final step of purification the active peak from anion-exchange chromatography was run in reverse-phase HPLC (Fig. 18a). The large peak containing the M_r 75000 protein was eluted at 45% buffer B (45% acetonitrile). According to SDS/polyacrylamide gel electrophoresis of the purified protein (Fig 18b, track 3) the 75000 M_r protein had been purified to homogeneity. Immunoblotting with anti-Sp23 demonstrated it's specific reaction with the purified protein.

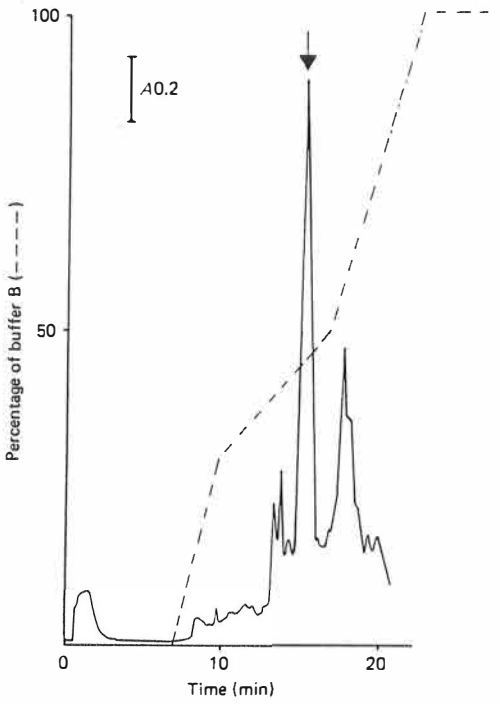

Figure 18. a) Reverse-phase h.p.l.c. chromatogram of the positive fraction of the anion-exchange run, b) SDS/polyacrylamide-gel electrophoresis of the M_r 75000 protein-containing peaks of the three sequential HPLC chromatography steps stained with Coomassie Brillianr Blue.

Immunoblotting with anti-sp23 antibodies was the only method available to monitore the purification steps of M_r 75000 polypeptide. The antigenic epitope of Mr 75000 was stable since the cross-reactivity with anti-sp23 antibodies also remained under denaturing conditions in immunoblotting, and in reverse phase cromatography. Anti-sp23 peptide antibodies are directed to a short linear epitope and the corresponding epitope in Mr 75000 protein may also be linear and independent of the tertiary structure of the protein.

In the later studies, the M_r 75000 was further characterized and named cytovillin (Pakkanen, 1987). This protein was cloned, and computer analysis of the cytovillin cDNA sequence made from EMBL DNA and protein data banks revealed no significant homology to other DNA and protein sequences (Turunen et al., 1989). The

highest homology between the original synthetic peptide and cytovillin was found to be 6/10 amino acids (Turunen et al., 1989) (Fig. 19). This may also be the antigenic epitope detected by the anti-sp23 antibodies.

```
sp23                              E D P S Q F Y E R L
                                  * * *   * *     *
Cytovillin  (residues (62-71)     E D P L Q F K F R A
```

Figure 19. Sequence homology between sp23 and probable antigenic epitope of cytovillin. The cDNA sequence of cytovillin is published by Turunen et al., 1989. Asterixes indicate matched amino acids.

Several laboratories have reported the cross-reactivity of anti-peptide antibodies between related proteins (Geysen et al., 1985; Närvänen et al., 1987) or even between unrelated proteins (Nigg et al., 1982). Comparison between polyclonal antibodies to viral proteins and anti-peptide antibodies to synthetic peptides derived from corresponding proteins shows that anti-peptide antibodies cross-react more readily than polyclonal antibodies (Geysen et al., 1985). In addition Geysen and coworkers have shown that in small antigenic epitopes (5 amino acids in length) only a few amino acids are essential for recognition and the others can be replaced without any effect on antibody recognition (Geysen et al., 1985).

It has been argued that if the surface area of a protein that is recognized by an antibody is of the order of 20x25Å, all protein epitopes are discontinuous to some extent, and that cross-reacting peptides mimic only the "primary" interaction site (Barlow et al., 1986). Only a few side-chains of the epitope can be in direct contact with the antibody molecule. If different protein molecules have a short sequence with amino acid side-chains having the same or similar physicochemical properties correctly positioned, cross-reaction with anti-peptide antibodies may be possible. Using mimotope design strategy, Geysen and coworkers have succeeded in building artifical complementary structures to monoclonal antibodies from amino acids. These antibodies have been directed to a discontinuous antigenic determinant on foot-and-mouth disease virus (Geysen et al., 1986).

4.2. The immunoreactive antigenic site of HIV-1 gp41 (II, III)

4.2.1. Computer analysis of gp41 of HIV-1

According to Eisenberg's hydropathy index derived with a seven amino acid window, the HIV-1 gp41 peptide A15 (residues 599-613; Fig. 20) is derived from a hydrophobic region. This region is conserved in six different HIV-1 isolates, but has two to three point mutations in six other HIV-1 isolates (Table 7).

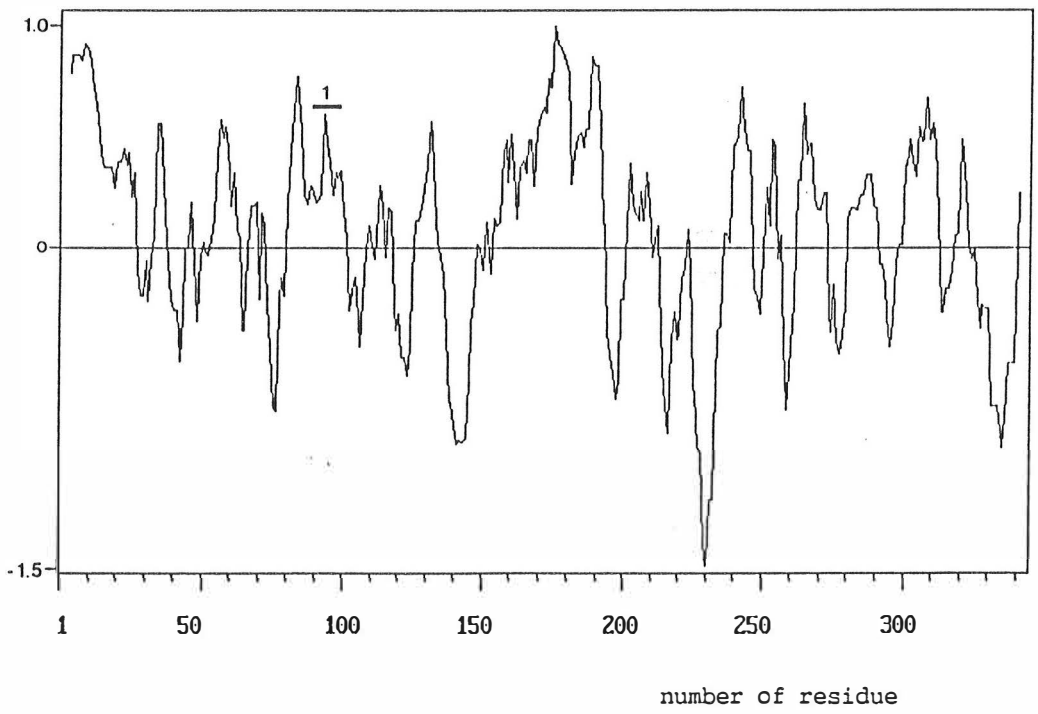

Figure 20. Hydrophobicity plot of the amino acid sequence of gp41 of HIV-1 using a window of 7 amino acids. Hydrophobic regions are above and hydrophilic regions are below the zero-line. Peptide A15 is marked with the bar 1.

Table 7. Sequence alignment of different HIV-1 and HIV-2 strains. Residues identical with the sequence shown at top are indicated by vertical lines (Human retroviruses and AIDS, 1989).

HIV-1	HXB2	SGKLICTTAVPWNAS
	BRU	| | | | | | | | | | | | | | |
	MN	| | | | | | | T | | | | | | |
	SC	| | | | | | | T | | | | | T |
	SF2	| | | | | | | | | | | | | | |
	CDC4	| | | | | | | | | | | | | | |
	WMJ2	| | | | | | | T | | | | | | |
	RF	| | | | | | | T | | | | | | |
	MAL	| | | H | | | F | | | S | |
	ELI	| | | H | | | N | | | S | |
	Z6	| | | | | | T | | | S | |
	Z321	| | | I | P | N | | | S | |
	JY1	| | | H | | | T | | | S | |
HIV-2	ROD	AFRQV | H | T | | | VND
	NIHZ	AFRQV | H | S | | | VND
	MM142	AFRQV | H | S | | | PNA
	K6W	AFRQV | H | S | | | PNA

It has been generally proposed that the primary reason for the antigenicity of some polypeptide chains is their surface exposure, as predicted by hydrophilicity and tertiary structure analyses (Hopp et Woods; 1981; Kyte et Doolittle; 1982; Tainer et al.,1984; Westhof et al., 1984; Novotny et al., 1986). However, the highly immunoreactive sequence of peptide A15 is located in a very hydrophobic region and contains only one charged amino acid residue (Lys-3). The theories mentioned above are based on the idea that all antigenic epitopes are located on the surface of the protein molecule. This premise may be true when antibodies are raised to isolated proteins. The situation apparently changes dramatically during natural viral infection, where viral proteins are processed and presented by accessory cells. Viral proteins lose their tertiary structures and antibody responses are raised to the polypeptide fragments of the original protein.

4.2.2. Purified human anti-peptide antibodies (II)

An HIV-1 positive serum pool from 10 patients was first tested with peptide A15 EIA, and with whole virus in solid-phase EIA (Fig. 21). The serum pool reacted in both EIAs, but the absorbance values in different serum dilutions in peptide A-15 were higher than in whole-virus EIA (Fig. 21a). The purified anti-peptide A15 Ig fraction reacted only with the synthetic peptide in EIA, but not in the whole virus EIA (Fig 21b). The peptide B18 EIA, reactive with 30% of HIV-1 positive human sera (data not shown), was used as a control. The anti-peptide A15 Ig fraction did not react with peptide B18.

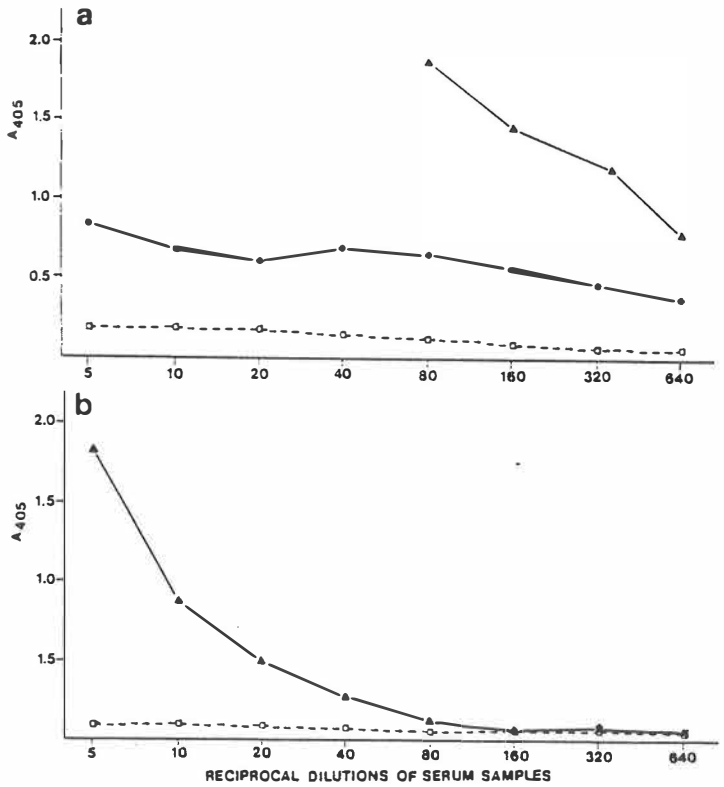

Figure 21. a) Reactivities of an unfractionated HIV-1 positive serum pool from 10 HIV-1 positive patients in whole virus EIA (●——●) and in peptide A15 EIA (▲——▲). In peptide A15 EIA the absorbance values at dilutions ‹1:80 were beyond the measuring range (›2.0). HIV-1-negative human serum did not react in peptide A15 EIA (□——□). b) Reactivities of affinity-purified anti-peptide A15 Ig fraction in the peptide A15 EIA (▲——▲). Purified anti-peptide Ig fraction did not react in the whole virus EIA or in in peptide B18 EIA (□——□).

As shown in Fig. 22 the original serum pool was immunoreactive with several HIV-1 proteins, including gp41 in immunoblotting and RIPA whereas the purified peptide A15 antibodies were unreactive with gp41 in both methods. Anti-peptide A15 antibodies did react with peptide A15-BSA complexes in immunoblotting.
In immunofluorescence (data not shown) with HIV-1 infected H9-cells, the original serum pool (diluted 1:50), showed strong staining, whereas the purified anti-peptide A15 Ig fraction (23 µg/ml) produced no staining.

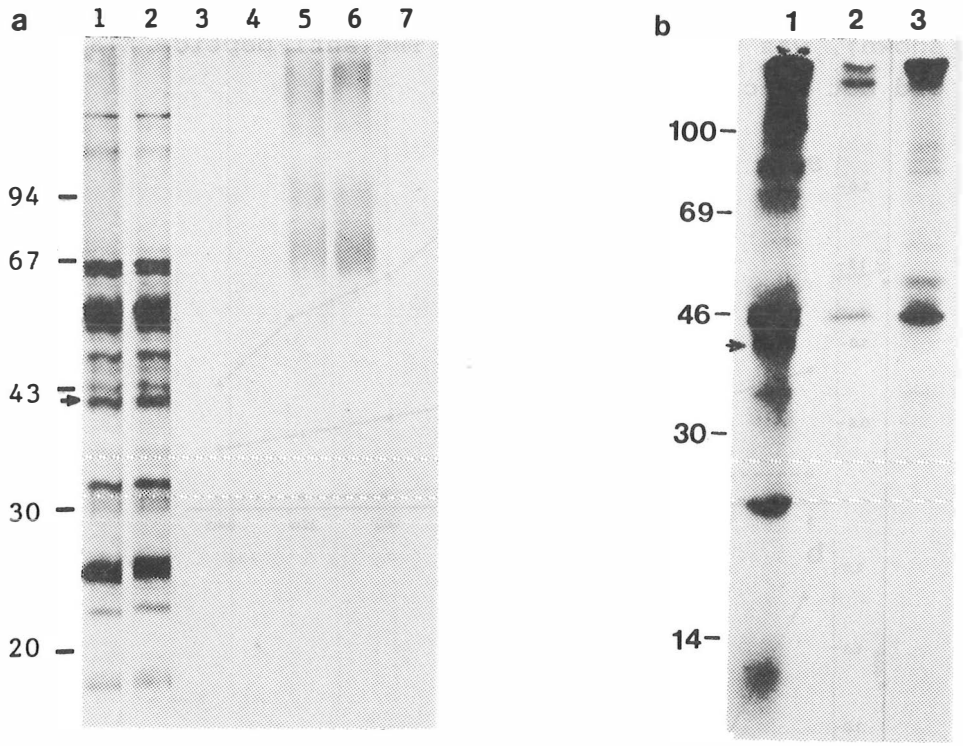

Figure 22. a) Immunoblot analysis of HIV-1 proteins and the peptide A15-BSA-conjugates blotted with different antibody samples. Lane 1, pool of unfractionated HIV-1 positive sera, diluted 1:100 and against viral proteins; Lane 2, column unbound antibodies against viral proteins; Lane 3, purified anti-peptide A15 Ig fraction, diluted 1:5 (5 µg/ml) against the viral proteins; Lane 4, column-unbound antibodies against peptide A15-BSA conjugate; Lane 5, purified anti-peptide A15 Ig fraction, diluted 1:5 (5 µg/ml) against peptide A15-BSA-conjugate; Lane 6, anti-BSA antibodies against peptide A15 conjugate; Lane 7, pool of HIV-1 negative human sera, diluted 1:100, against viral proteins.
b) Radioimmunoprecipitation assay of metabolically labelled HIV-1 proteins by unfractionated HIV-1 positive sera (Lane 1) by purified anti-peptide A15 Ig fraction (Lane 2) and by HIV-1-negative human sera (Lane 3).

These results indicate that the higly immunoreactive antigenic site in the transmembrane protein gp41 of HIV-1 remains undetectable with antibodies from individuals infected with HIV-1 when conventional immunochemical methods are used. It has been proposed that hydrophobic amino acid side chains are buried in the protein molecule (Chotia 1976). The hydrophobic nature of the peptide A15 and the unreactivity of human peptide antibodies with entire gp41 protein support the idea that the immunoreactive A15 epitope is partially or totally buried within intact gp41. This epitope may become exposed during antigenic prosessing and presentation by the accesory cells, during the HIV-1 infection. An alternative explanation is that all the immunochemical methods that have been used in this study may alter the structure of gp41, so that this antigenic determinant, exposed in the native gp41 in vivo, is hidden inside the denatured protein. Norrby and coworkers have shown that monoclonal antibodies directed to the amino terminal part of the synthetic peptide (gp41; amino acids 582-604) do not detect entire gp41 in EIA or in westernblotting. Monoclonal antibodies to central part of the peptide and rabbit polyclonal antibodies to entire peptide did detect whole gp41 in both tests. These results indicate that this epitope region is partially puried in the gp41 molecule (Norrby et al. 1989).

4.2.3. Peptide A15 EIA

Peptide A15 was further tested as a BSA complex in solid-phase enzyme immunoassay. The cut off value (4.2 EIU) was calculated according to the mean value of EIUs of 1,000 HIV-1 antibody-negative blood donors. The mean of 1000 samples was 0.9 EIU and the standard deviation (+1 SD) 1.2 EIU. The cut off value (4.2 EIU) was determined to be two times the mean value plus two standard deviations. None of the blood donor sera gave EIA values above the cut off value. In the cohort of 144 HIV-1 infected individuals, which were postive in whole-virus EIA and immunoblotting, 143 had EIU values over the cut off level, and 43% had values over the measuring range (150 EIU) (Fig 23).

This serum panel was further classified according to the stage of HIV-1 infection, and each group was compaired (Fig. 24). The A15 peptide EIA detected all 94 asymptomatic patients (ASX), 28 of the 29 patients with LAS, all ARC patients, and all AIDS patients studied. There was a tendency toward higher values in asymptomatic HIV-1 infected individuals, but patients with lymphadenopathy syndrome (LAS) or AIDS-related complex (ARC) also had values over the measuring range. In the group of AIDS patients only two cases were over the measuring range and relatively more cases had low EIU values than in other groups. Out of five known HIV-2 cases all were negative in the A15 peptide EIA.

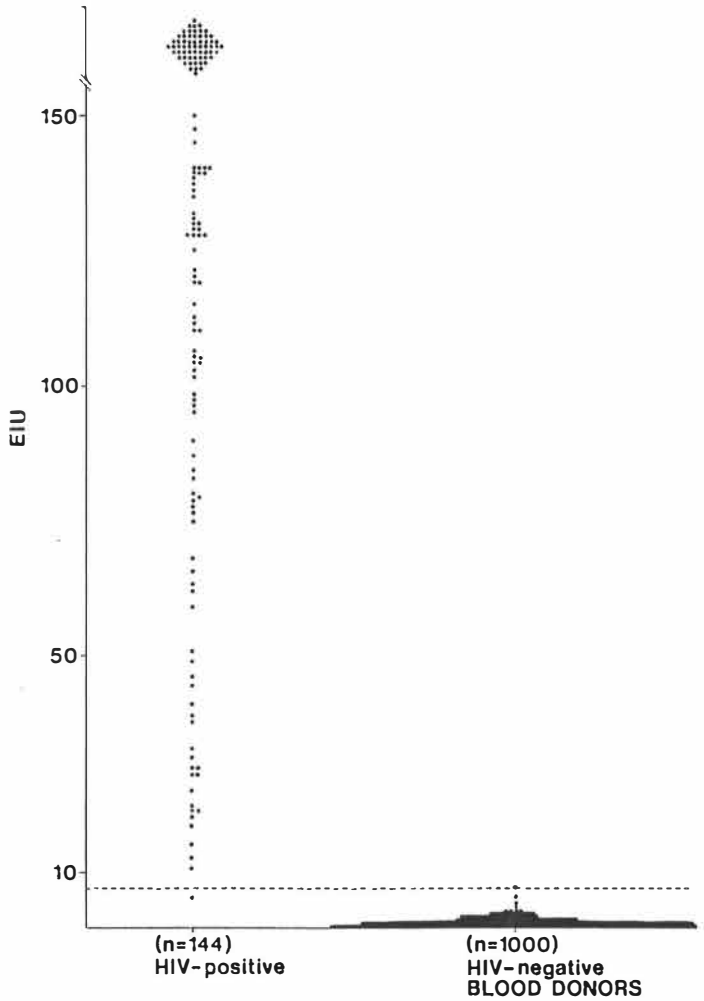

Figure 23. Reactivity of the peptide A15 EIA in the reference population and in patients with HIV-1 infection. The cut off level, indicated by the broken line, was calculated as twice the mean value of negative sera plus two standard deviations.

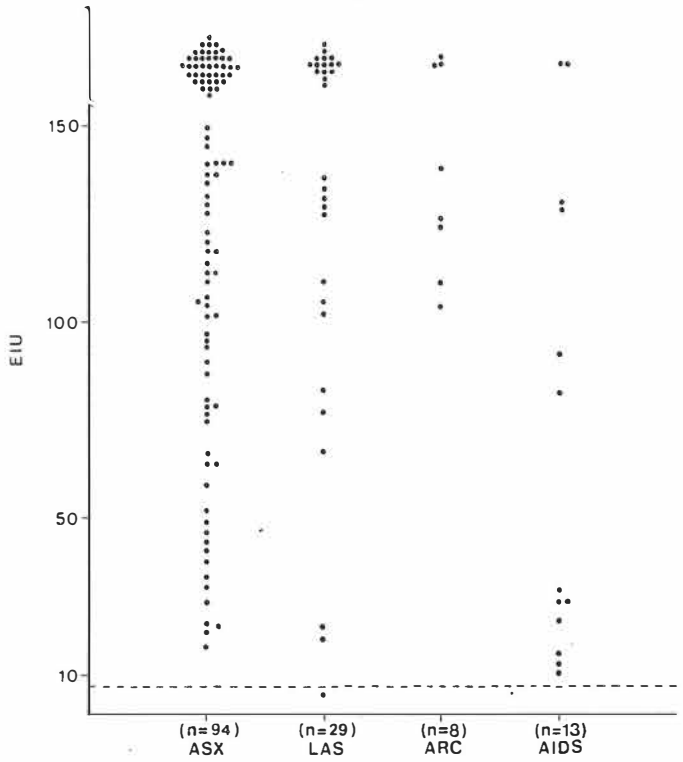

Figure 24. Reactivity of the peptide A15 EIA in different stages of HIV-1 infection. The cut off value was calculated as above.

The sensitivity of the peptide A15 EIA in the early stage of HIV infection was tested with eight serial serum sample panels collected during the seroconversion. Two of the cases were detected earlier as positive with the peptide EIA than with whole-virus EIA (panels 1 and 8, Fig. 25, Table 8).

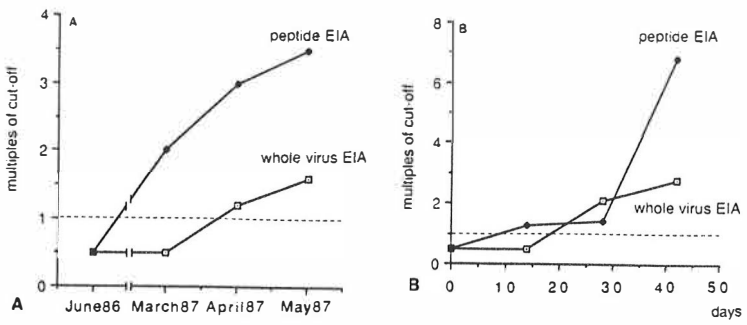

Figure 25. Comparison of the sensitivity of the A15 peptide EIA with whole-virus EIA in sera of two patients studied prospectively during the course of HIV-1 infection. The second sample in A was positive for antibodies to p24, and p55 in WB.

Table 8. Detection of HIV-1 infection in eight patients by the A15 peptide EIA, whole virus EIA and immunoblotting. In immunoblotting, antigen strips were prepared by DuPont.

Patient no and dates	A15 peptide EIA	Whole-virus EIA	Immuno-blot
1 1-6-86 (ASX)	-	-	-
1-3-87 (ASX)	+	-	p24,p55
1-4-87 (ASX)	+	+	gag,env
2 26-6-85 (ASX)	-	-	-
8-4-86 (LAS)	+	+	gag,env
8-4-87 (LAS)	+	+	gag,env
3 31-7-85 (ASX)	-	-	-
12-11-86 (ASX)	+	+	gag,env
11-11-86 (LAS)	+	+	gag,env
4 12-6-84 (ASX)	-	-	-
12-12-84 (ASX)	+	+	gag,env
27-3-85 (LAS)	+	+	gag,env
5 27-3-84 (ASX)	-	-	-
7-11-84 (ASX)	+	+	p24,gp120
17-7-85 (ASX)	+	+	gag,env
6 21-11-84	-	-	-
6-11-85	+	+	gag,env
7 22-5-84 (ASX)	-	-	-
2-1-85 (ASX)	+	+	gag,env
26-6-85 (LAS)	+	+	gag,env
8 20-10-86 (ASX)	-	-	-
2-11-86 (ASX)	+	-	p24
17-11-86 (ASX)	+	+	gag,env

Out of 162 sera from different stages of HIV-1 and idetified positive in immunoblotting, 161 were positive in peptide EIA and 156 positive in whole-virus EIA (Table 9). Seven sera positive in peptide EIA did not react with gp41 in immunobloting, but detected p24, p51 and gp120.

Table 9. Comparison of the sensitivity of the peptide A15 EIA with whole-virus EIA and immunoblotting in detection of antibodies to gp41 during different stages of HIV-1 infection. In immunoblotting (WB), antigen strips prepared either by DuPont of Labsystems were used with identical results.

Patient with HIV-1 infection	No.	Positive in A15 peptide EIA	Positive in whole-virus EIA	Reactive with gp41 in WB
Asymptomatic	106	106	100	97
LAS	35	34	35	33
ARC	8	8	8	8
AIDS	13	13	13	13
total	162	161	156	151

The specificity of the peptide A15 was compared to whole-virus EIA with the serum panels from patients with different clinical disorders unrelated to HIV-1 infection. In the group of miscellaneous infections that gave easily false-positive reactions in whole-virus EIA (n=50), only two patients gave false-positive reactions with the peptide A15 EIA (Table 10).

Table 10. Comparison of the specificity of the peptide A15 EIA with whole-virus EIA in patients with different clinical disoders but no HIV-1 infection.

Patient group	Positive in A15 peptide EIA (%)	Positive in whole-virus EIA (%)
Kidney Transplantation patient under Hemodialysis	0/20 (0)	0/20 (0)
Miscellaneous infections	2/50 (4)	13/50 (26)

The peptide A15 EIA contains only one epitope from the highly immunodominant region of gp41. This epitope was able to detect antibodies from patients in different stages of HIV-1 infection. Only one serum, from a patient with LAS, was negative. In addition, the peptide A15 EIA was more sensitive in the early stage

of HIV-1 infection than the whole-virus EIA. Antibodies to A15 epitope rise early and are detectable during the the course of HIV-1 infection. Seven sera were positive in peptide EIA, but did not react with gp41 in immunoblotting. This data further support the idea that there is a structural hindrance to this epitope in the tertiary structure of gp41 when whole protein is used as antigen in the antibody tests.

In the panel of 1000 HIV-1 antibody negative blood donors, the specificity was 100% and the overall specificity of the test for HIV-1 infection was 99.8%. The specificity is higher in peptide EIA than in whole-virus EIA in this study. In the peptide A15 EIA, the antigen is homogeneous and free from cellular contaminants which may give false-positive results in whole-virus EIAs (Jackson & Balfour 1988).

4.3. Characterization of a neurite outgrowth-promoting domain of laminin (IV)

4.3.1. Hydrophobic moment plot of the laminin B2 chain

Laminin has a cross-like structure in rotary shadowing electron microscopy (Engel et al., 1981). The arms of the cross are probably formed from coiled coil alpha-helical structures (Paulsson et al., 1985). According to this prediction, we used a 100 degree angle when calculating the amphipathic nature of the B2 fragment. The position of the amphipathic maximum (μ=0.81) locates near the carboxy terminus of the polypeptide (Fig 26a). Two peptides p20 (amino acids 185-194) and its longer derivate p32 (amino acids 175-204) were synthesized from this region. The hydrophobicity curve derived with a 4 amino acid window shows that peptide p20 is not located in totally hydrophobic region (Fig 26b), but slight periodicity can be seen. This may support the result showing the amphipathic nature of peptide p20.

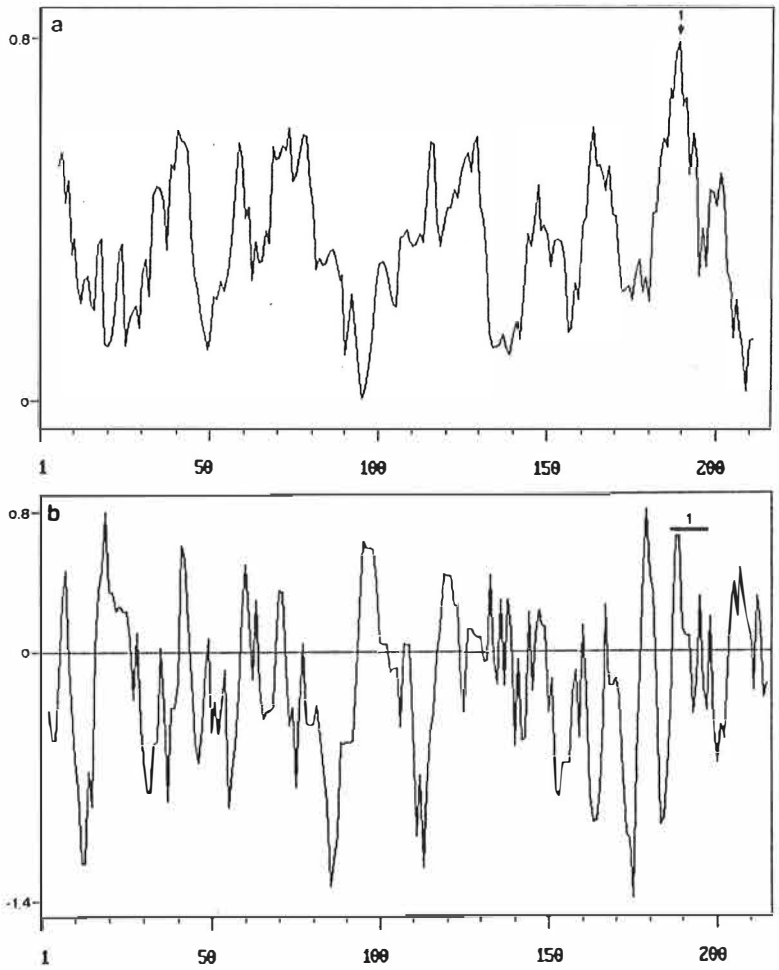

Figure 26. a) Hydrophobic moment plot of a portion of the laminin B2 chain. Peptide p20 is marked with the arrow number 1. b) Hydrophobicity curve of B2 fragment. Peptide p20 is marked with bar number 1.

4.3.2. Biological assays of the peptides.

The biological activity of the synthetic peptides were tested in two ways. First, the peptides were used as inhibitors of cell attachment and neuronal outgrowth. Further, the promoting activity of the peptides was tested in cell and organotypic cultures. Previously it has been demonstrated that the cell-attachment activity could be substituted by a synthetic peptide from the P1 fragment (Graf et al. 1987a). These authors further report that their peptide sequence supported neuronal attachment but no neurite outgrowth. Peptide P31 was selected according to these results.

4.3.2.1. Inhibitory effects of the peptides

The specific inhibitory effect of the laminin peptides were tested in the cultures of newborn rat cerebellar neurons or mouse embryo DRG, plated on laminin-coated glass coverslips. The cultured cells extended long neurites within 12 h (Fig. 27a,c). Neuronal death occured (Fig. 27b), and neurite outgrowth in the DGR cultures was inhibited within 12 h (Fig. 27d) when 100 µM (100 µg/ml) of peptide p20 or its longer derivate p32 was added simultaneously with the cells. The cell-attachment peptide p31 or control peptides P1-P7 did not have any inhibitory effect in similar concentrations (up to 200 µg/ml). Time-lapse video recording revealed that addition of 50 µg/ml of peptide p20 to the culture medium of neurons that had already extended neurites on laminin caused complete cessation of cytoplastic streaming and movement of the growth cones and ruffling membranes within 2 h exposure to peptide p20 (not shown). The inhibitory effect of the peptide for neuronal survival was dose dependent (Fig. 28), only concentrations over 50 µg/ml caused specific cell death. The toxicity was specific for primary neurons, and peptide p20 concentrations up to 200 µg/ml did not have any effect on primary glial cells or on differentiation of 11-day-old mouse embryo kidney (not shown).

Non-toxic concentrations of p20 (e.g.,1-5 µg/ml; 1-5 µM; see Fig. 28) were further tested in competition assays. Rat cerebellar neurons were plated on laminin simultaneously with peptide p20 in culture medium. The attachment of neurons to laminin was reduced down to 55-70% of that seen on native laminin, and outgrowth of long neurites was inhibited down to 20-40% of that seen on native laminin (Fig. 29).

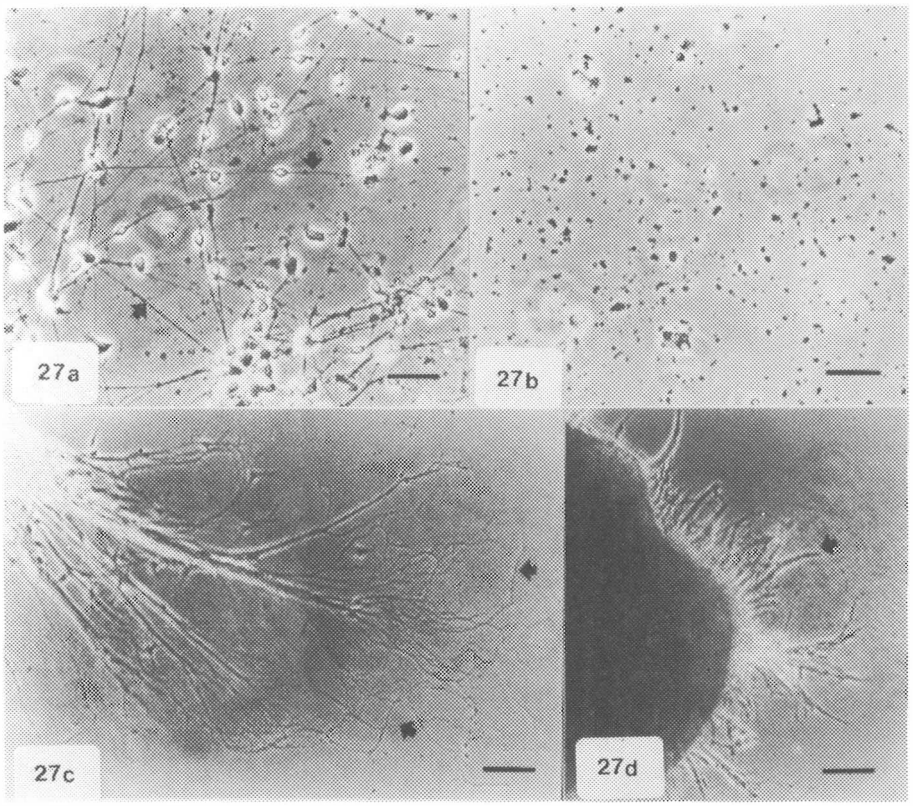

Figure 27. Phase-contrast micrographs of newborn rat cerebellar (a,b) and 16-day-old mouse embryo dorsal root ganglia (c,d) neurons grown on laminin in the absence (a,b) or in the presence (b,d) of 100 µg/ml (100 µM) of peptide p20. Neurons showed extensive neurite outgrowth on laminin (a,c, arrows). In the presence of 100 µg/ml of the peptide p20 (or p32), massive cell death occurred (b) and all neurite outgrowth was inhibited (d, arrow). Bar = 40 µm.

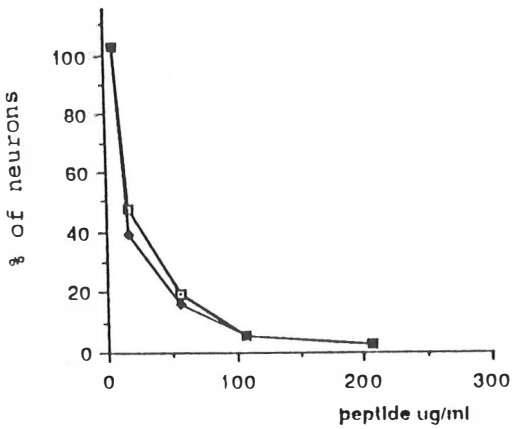

Figure 28. A dose-response curve of the effect of peptide p20 added to the culture medium on the number of neurons (□) and neurites (■) in cultures of newborn rat cerebellum grown on laminin. Calculated neurons in cultures without peptide in the culture medium = 100%. Concentrations higher than 50 µg/ml caused a specific toxic effect.

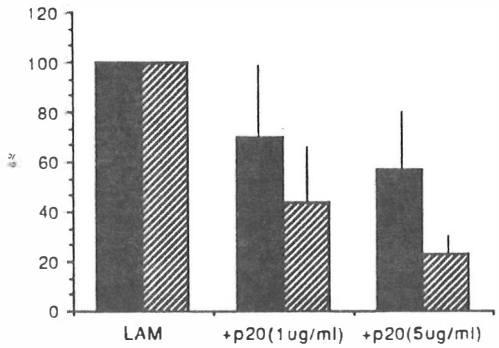

Figure 29. Attachment of neurons (■) and outgrowth of neurites (▨) in cultures of rat cerebellar neurons grown on laminin in the presence of 1-5 µM (1-5 µg/ml) of free peptide p20 in the culture medium. The most clear cut effect is the ability of peptide p20 (5 µg/ml) to inhibit outgrowth of neurites on the laminin down to 30% of the amount observed in the absence of the peptide (LAM).

4.3.2.2. Neurite outgrowth-promoting and cell-attachment activity of the peptides

The neurite outgrowth promoting activity of free peptide p20 and its longer derivative p32 was tested in organotypic cultures at non-toxic concentrations (100 nM; 0.1 µg/ml). Both peptides showed extensive neurite outgrowth through the pores of the filter within 24 h in the cultures of newborn mouse spinal cord and cerebellum (Fig 30 B,D) and lower stimulus in embryonic DRG cultures (Fig. 30 F). The cultures without any peptides (Fig. 30 A,C,E) showed only slight outgrowth of short neurites. The cell-attachment peptide p31 or seven other control peptides (P1-P7) did not promote neurite outgrowth. Laminin at 100 nM (100 µg/ml) concentration in culture medium showed comparable neurite outgrowth promoting activty to peptide p20 (Fig. 30 G). Higher concentrations of p20 (up to 100 µg/ml) did not improve or inhibit the neurite outgrowth response.

For cell-attachment and neurite outgrowth studies in cell cultures, glass coverslips were coated with p20-BSA and/or p31-BSA at 100-200 nM (100-200 ng/ml) and an equimolar concentration of native laminin (100-200 µg/ml; 100-200 nM). BSA substratum was used as a control at a concentration of 600 ng/ml, which is equivalent to the BSA concentration of the peptide conjugates

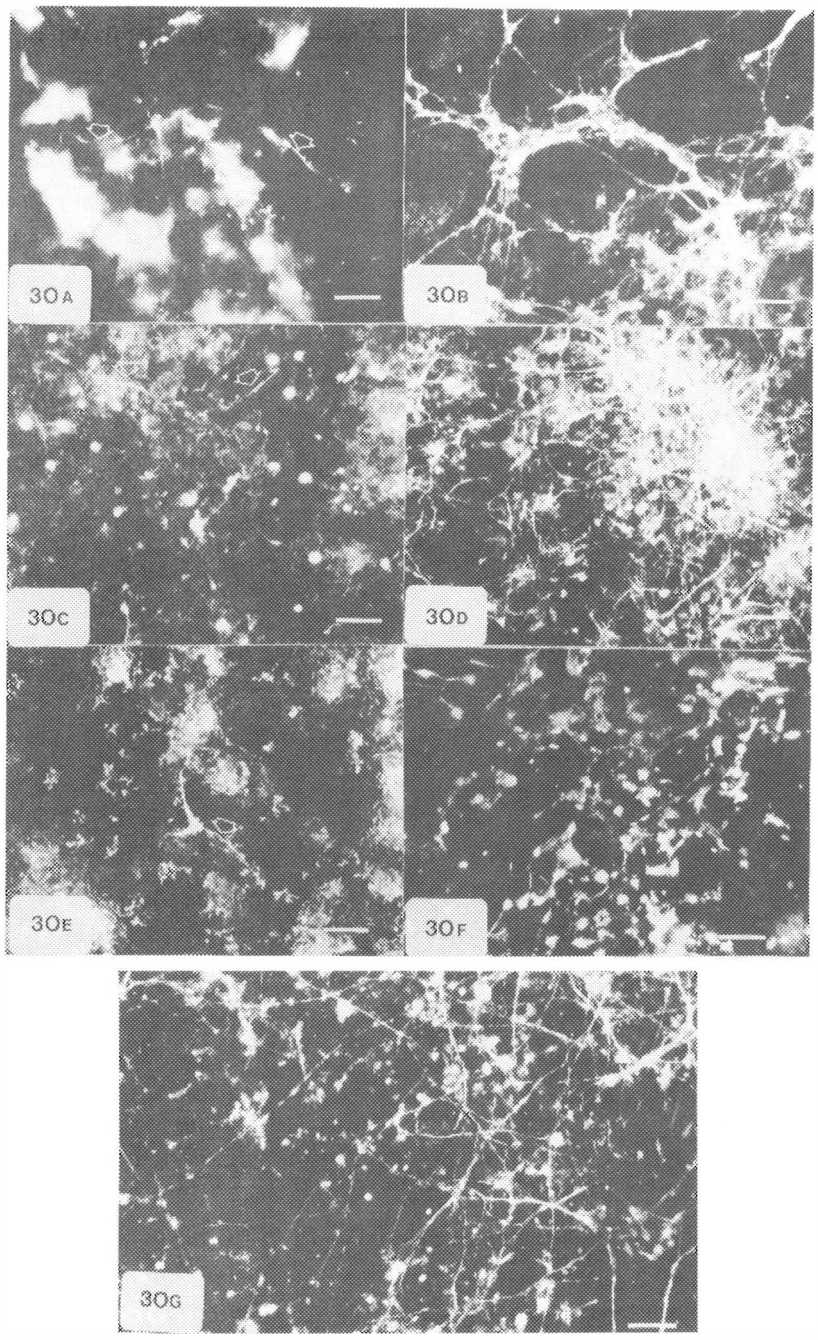

Figure 30. Demonstration of neurofilament protein immunoreactivity in fluorescence microscopy in organotypic cultures of newborn mouse spinal cord (A,B), cerebellum (C,D,G) and 16-day old mouse embryo DGR (E,F). The fibers grown through the filter in 24 h are demonstrated in the absence (A,C,E) or in the presence (B,D,F) of 100 ng/ml (100 nM) of peptide p32 or laminin (G; 100 µg/ml = 100 nM) in the culture medium. The presence of peptide p32 or laminin stimulated outgrowth of neurites in all areas (cf. A,C,E with B,D,F,G). In cultures of DRG (F) the effect of peptide p32 was less dramatic than with central neurons. Bar = 20 µm.

used at 200 ng/ml. Rat cerebellar neurons were plated on coated coverslips and were grown for 12 h. For comparisons, attachment and neurite outgrowth on native laminin was considered as 100%. The peptide p20-BSA (200 ng/ml) alone supported neuronal attachment nearly as well as the peptides p20-BSA (100 ng/ml) and p31-BSA (100 ng/ml) used simultaneously as culture substratum (cf. p20 with p20 + p31 in fig. 31). Outgrowth of neurites on p20-BSA alone (p20 in fig. 31) was up to 60% of that seen on native laminin, and was comparable to the neurite outgrowth when the peptides p20-BSA and p31-BSA were used simultaneously (see p20-p31 in fig. 31). Peptide p31-BSA (200 ng/ml, p31 in fig 27) alone supported attachment of neurons, but its effect on outgrowth of neurites was only marginally better than that of albumin (cf. BSA and p31 in fig. 31).

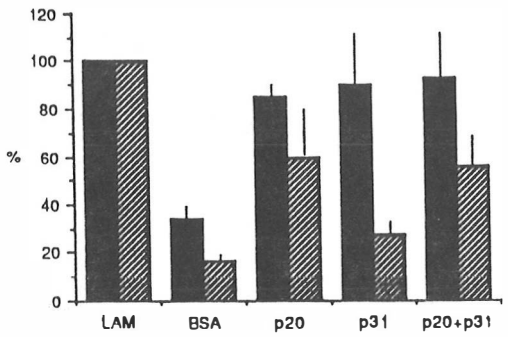

Figure 31. Neuronal attachment (■) and number of neurites (▨) in cultures of rat cerebellar neurons grown on laminin (LAM) (coating concentration 100 µg/ml), BSA (coating concentration 0.6 µg/ml), p20-BSA (coating concentration 0.2 µg/ml), p31-BSA (coating concentration 0.2 µg/ml) or p20-BSA + p31-BSA (coating concentration 0.1 µg/Ml each). The peptide p20-BSA alone was comparable in neuronal attachment and neurite outgrowth to the two peptides p20-BSA/p31-BSA together. The peptide p31-BSA alone did not support neurite outgrowth any better than albumin, although it supported attachment of neurons.

In the inhibitory tests, peptide p20 and its longer derivative showed specific toxic effect for neurons at concetrations over 50 uM. We were not able to test if native laminin had the same kind of effect, since equimolar concentrations of laminin (50 µM; 50 mg/ml) would not remain in solution. However, many biologically active molecules, such as insulin, are toxic at high concentrations. In the competition assays, peptide p20 or p32 concentrations below the toxic range (1-5 µM) peptides p20 or p32 did not inhibit totally neurite outgrowth, suggesting that

there may be more than one neurite outgrowth promoting domain in laminin. This was further tested in cell cultures with peptide-BSA conjugates as substratum for neurons. Peptide p20-BSA conjugate alone or together with P31-BSA supported neuronal attachment and neurite outgrowth, but did not fully simulate the effect of native laminin. In organotypic cultures peptides p20 or p32 were able to stimulate neurite outgrowth as well as native laminin in equimolar concentrations, indicating that the effect of the p20 domain is independent of other possible neurite outgrowth promoting domains.

Later studies with synthetic peptides have also demonstrated neurite outgrowth promoting activity in the carboxy terminal part of the A-chain (Tashiro et al. 1989). A pentapeptide IKVAV was shown to be active in cell-adhesion and neurite outgrowth. Neurite outgrowth domains in A and B2 chains are located in the same area in native laminin, indicating that the neurite outgowth promoting activity of laminin is in the long arm of the molecule near the globule (Fig 32).

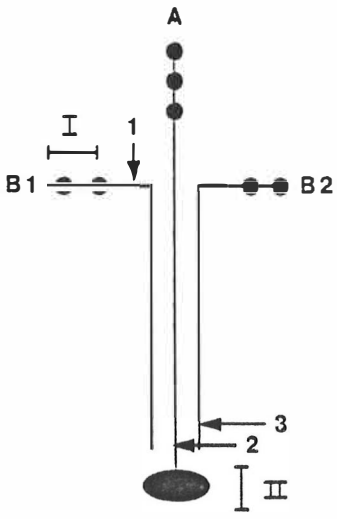

Figure 32. Schematic representation of the laminin molecule and location of the sequences of active peptides. 1) Cell attachment site (Graf et al. 1987). 2) Neurite outgrowth promoting site of A-chain (Tashiro et al. 1989). 3) Neurite outgrowth promoting site of B2-chain (Liesi et al. 1989). Interaction sites are marked with roman numerals. I) Collagen binding site (Charonis et al. 1986). II) Heparin binding site (Ott et al. 1982). Black balls indicate the globular regions of the laminin molecule.

5. Concluding remarks

Synthetic peptides provide excellent tools to study protein interactions at individual amino acid level. The continuous epitope regions, mimicked with synthetic peptides, are idependent of proteins tertiary structure. Antibodies elicted to synthetic peptides can be used as a probes to study novel proteins.

In this study synthetic peptides have been used to characterize immunoreactive antigenic epitope from the transmembrane protein gp41 of HIV-1, and to localize laminin outgrowth promoting activity. Anti-peptide antibodies have been used to monitor the purification of a novel protein. Properties of the studied peptides were examined using predictive algorithms based on different physicochemical properties of the individual amino acids. The present study show:

1. Antibodies to the peptide (sp23), derived from the cDNA of human endogenous retrovirus, detected a protein of M_r 75 000 in immunoblotting. This protein was purified using conventional immunochemical methods and the purification steps were monitored with anti-sp23 antibodies in immunoblotting. Later this protein was cloned and named cytovillin (Pakkanen et al. 1988, Turunen et al. 1989). The discovery of cytovillin was based on the cross-reactivity of polyclonal anti-peptide antibodies to "unexpected" protein indicating that "artifical" anti-peptide antibodies may have broad specificty.

2. Synthetic A15 peptide, derived from the transmembrane protein gp41 of HIV-1, showed strong reactivity with antibodies from individuals infected with HIV-1. Affinity-purified human anti-A15 antibodies did not react with entire gp41 molecule in conventional immunochemical methods. This finding was further

supported with seven sera from individuals with HIV-1 infection and being non-ractive to gp41 in immunoblotting, but highly immunoreactive with A15 peptide. These results suggest that A15 epitope is partially or totally buried in the molecule.

3. Peptide A15 was used as antigen in indirect solid-phase EIA. The sensitivity of the EIA to detect HIV-1 infection was studied with human sera from individuals in different stages of HIV-1 infection. The results showed high sensitivity for the A15 peptide as an antigen to detect HIV-1 infection especially in the beginning of seroconversion. The EIA was more specific than whole-virus EIA with sera from individuals with miscellaneous infections but not with HIV-1 infection.

4. Synthetic peptide p20 and its longer derivative p32, derived from B2 subunit of laminin, showed specific inhibitory effect to neuronal outgrowth and cell attachment in cell cultures. In organotypic cultures peptide p20 showed equal neuronal outgrowth promoting activity than purified laminin. In addition the cell-attachment activity of peptide p31 published by Graff et al. (1987) was confirmed. The neuronal outgrowth promoting activity of B2 was localized to same region into the long arm of laminin molecule than the other epitope from subunit A (Tamashiro et al. 1989).

5. The computer analyses showed that the immunoreactive epitope A15 of transmembrane protein gp41 of HIV-1 is located in a hydrophobic region, and that the neurite outgrowth promoting activity is located in the amphipatic maxum of B2 subunit of laminin.

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This work is dedicated to my family, especially to my wife Outi, for her patience and encouragement.

Helsinki, November 1990

Ale Närvänen

Selostus

Synteettiset peptidit proteiinin aktiivisten kohtien mallina ja antigeenisenä epitooppina

Synteettisiä peptidejä voidaan käyttää malleina paikallistettaessa aktiivisia kohtia ja antigeenisia epitooppeja proteiinien primaarirakenteesta. Synteettisillä peptideillä voidaan mallittaa jatkuvia epitooppeja, jotka ovat riipumattomia proteiinin kolmiulotteisesta rakenteesta. Eläimissä tuotettuja vasta-aineita synteettisiä peptidejä vastaan voidaan käyttää alkuperäisen proteiinin tutkimiseen. Tässä työssä on käytetty synteettisiä peptidejä sekä karakterisoimaan HIV-1 viruksen transmembraaniproteiinin gp41:n immunorektiivista epitooppia että paikallistamaan lamiiniinin hermosäikeiden kasvua aktivoivaa epitooppia. Kanin vasta-aineita synteettistä peptidiä vastaan on käytetty uuden proteiinin puhdistuksen seurantaan. Tutkimuksissa käytettyjen peptidien fysikokemiallisia ominaisuuksia on tutkittu tietokonemalleilla. Tulokset osoittavat seuraavaa:

1. Kanissa tuotettu vasta-aine synteettistä peptidiä vastaan, joka on johdettu ihmisen endogeenisen retroviruksen geenisekvenssistä, tunnisti 75 000 kD suuruisen proteiinin ihmisen koriokarsinooma solulinjasta immunoblottausmenetelmällä. Proteiini puhdistettiin sarjalla useita eri biokemiallisia menetelmiä käyttäen peptidi vasta-ainetta proteiinin puhdistuksen seurantaan. Myöhemmissä tutkimuksissa tämä proteiini kloonattiin (Turunen 1989) ja nimettiin sytovilliiniksi (Pakkanen 1988). Proteiinin löytyminen perustui peptidi-vasta aineen ristireaktioon sytovilliinissä olevan samankaltaisen epitoopin kanssa.

2. Synteettinen peptidi A15, joka on johdettu ihmisen immuunikato viruksen (HIV-1) membraaniproteiinin gp41:n aminohappoketjusta, reagoi voimakkaasti HIV-1 infektoituneiden potilaiden vasta-aineden kanssa. Nämä vasta-aineet puhdistettin affiniteettikromatografisella mentelmällä. Puhdistetut vasta-aineet eivät reagoineet kokonaisen gp41 proteiinin kanssa eri immunologisissa testeissä. Seitsemän seerumia eri HIV-1 postiivista potilaista, jotka olivat peptidi A15 positiivisia eivät reagoineet gp41:n kanssa immunoblottauksessa. Tulokset viittaavat siihen, että vastaava antigeeninen epitooppi on kiertyneenä osittain tai kokonaan proteiinin sisäosiin estäen vasta-aineiden sitoutumisen.

3. Peptidi A15:ta käytettiin antigeenina epäsuorassa EIA menetelmässä. Menetelmällä mitattiin vasta-aineita potilaista, jotka olivat eri HIV-1 infektion vaiheissa. Tulokset osoittivat, että peptidiin perustuva EIA on herkkä, varsinkin kun mitattiin infektion alkuvaiheita (serokonversio). Peptidi A15 EIA osoitti parempaa spesifisyyttä kuin kokovirus EIA testattaessa seerumeita HIV-1 negatiivisista potilaista, joilla oli eri tyyppisiä infektioita. Nämä tapaukset antoivat useammin väärän positiivisen tulokseen kokovirus EIA:ssa kuin peptidi A15 EIA:ssa.

4. Laminiini on rakenneproteiini, johon hermosolut sitoutuvat ja joka stimuloi neuronien kasvua. Hiiren laminiini B2 alayksiköstä johdettu synteettinen peptidi p20 ja sen pitempi versio p32 esti spesifisesti neuronien kasvua hermosoluviljelmissä. Korkeina annoksina peptidit olivat toksisia. Hermokudosviljelmissä peptidi p20 stimuloi neuronien kasvua yhtä hyvin kuin puhdistettu laminiinimolekyyli.

5. Matemaattiset mallit epitooppien rakenteesta osoittivat, että immunoreaktiivinen peptidi A15 sijaitsee hydrofobisella alueella. Laminiinin neuroneja stimuloiva peptidi sjaitsee laminiinin B2 alayksikön amfipaattisessa maksimissa.

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ORIGINAL PAPERS

I

PURIFICATION, FROM CULTURED CHORIOCARCINOMA CELLS, OF A 75000-Mr PROTEIN REACTING WITH ANTIBODIES TO A SYNTHETIC PEPTIDE BASED ON CLONED HUMAN ENDOGENOUS PROVIRUS NUCLEOTIDE SEQUENCE

Biochem. J. 231:53-57, 1985

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Ale Närvänen

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II

HIGHLY IMMUNOREACTIVE ANTIGENIC SITE IN A HYDROPHOBIC DOMAIN OF HIV-1 gp41 WHICH REMAINS UNDETECTABLE WITH CONVENTIONAL IMMUNOCHEMICAL METHODS

AIDS 2:119-123, 1988

by

Ale Närvänen, Mirja Korkolainen, Sari Kontio, Jukka Suni, Saija Turtiainen, Paul Partanen, Jozsef Soos, Antti Vaheri & Marja-Liisa Huhtala

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III

SYNTHETIC env gp41 PEPTIDE AS A SENSITIVE AND SPECIFIC DIAGNOSTIC REAGENT IN DIFFERENT STAGES OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION

J.Med.Virol. 26:111-118, 1988

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IV

IDENTIFICATION OF A NEURITE OUTGROWTH-PROMOTING DOMAIN OF LAMININ USING SYNTHETIC PEPTIDES

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Päivi Liesi, Ale Närvänen, Joszef Soos, Hannu Sariola &
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