CHARLES UNIVERSITY PRAGUE
FACULTY OF SCIENCE
DEPARTMENT OF BIOCHEMISTRY

FLUORESCENCE LABELING OF NK CELL RECEPTORS FOR BINDING STUDIES

Bachelor Thesis

Supervisor: Doc. RNDr. Karel Bezouška, CSc.

Prague 2006

Georgia Christofi

Declaration

I declare that this thesis is entirely my own work under the guidance of my supervisor doc. RNDr. Karel Bezouška, CSc. and all the information included in this work has been considered carefully.

Prague, 22nd August 2006 Signature.....

Preface

A lot of attention was aimed at the research of lectin receptors of NK cells in the recent years. NK cells play an important role in whole immune system. The results obtained from the research of lectin receptors structure, binding characteristics, and connection of these receptors to NK cells are used in medical treatment. In Czech Republic, there is a working group focused on this research. This group, formed as a cooperation between the Department of Biochemistry of Charles University Prague and the Laboratory of Molecular Architecture at the Institute of Microbiology of Academy of Science of Czech Republic. A large amount of new information about NK receptors was discovered by this team. I would like to thank this working group for the support during the preparation of my bachelor thesis, which is focused on structure of lectin-like receptors of NK cells, especially rat receptor NKR- P1.

Acknowledgement

I would like to thank everybody who helped me one way ore another with this bachelor thesis. Special thanks to my supervisor doc. RNDr. Karel Bezouška, CSc. for everything he has taught me and for all his support during this work. Moreover, special thanks to all my colleagues for their help, understanding and patience. Finally, I would like to thank all my friends and especially my parents for their moral and material support during my studies. To them I dedicate this work.

Thank you

Contents

List of abbrevations:

ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cells
BSA	Bovine serum albumin
BCR	B-cell receptors
CBB	Coomassie Brilliant Blue
CRD	Carbohydrate recognition domain
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DTT	Dithiothrietol
Fab	Fragment antigen binding
Fc	Fragment constant
FITC	Fluorescein isothiocyanate
IFN-α	Interferon alfa
IFN-γ	Interferon gamma
ITIM	Immunoreceptor tyrosine-based inhibitory motif
MHC	Major Histocompatibility Complex
MIP-1	Macrophage inflammatory protein-1
NHS-Fluorescein	5-(and 6-)carboxyfluorescein succinimidyl ester
NHS-Rhodamine	5-(and 6)-carboxytetramethylrhodamine succinimidyl ester
NK	Natural Killer cells
NKC	Natural Killer Cluster
NKR-P1A	Natural Killer Rat Protein A
NKR-P1B	Natural Killer Rat Protein B
PBS	Phosphate Buffered Saline
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PMN	Polymorphonuclear leukocytes
RANTES	Regulated on activation,normal T expressed and secreted
SDS	Sodium dodecyl sulfate
SHP-1,SHP-2	Intracellular tyrosine phosphatases
TCR	T-cell receptors
TEMED	N,N,N',N'-tetramethylethylendiamine
Tris	Tris(hydroxymethyl)aminomethane

1 Theoretical introduction

1.1 Immune System and its Components

1.1.1 Immune system

The human immune system is a truly amazing constellation of responses to attacks from outside the body. It has many faces, a number of which can change to optimize the response against these unwanted intrusions. It is composed of many interdependent cell types that collectively protect the body from bacterial, parasitic, fungal, viral infections and from the growth of tumor cells. The cells of the immune system can engulf bacteria, kill parasites or tumor cells or kill virally infected cells.

1.1.2 Cells of the immune system

For the normal function of the immune system, a variety of different cells is needed. They are the T-cells: T-lymphocytes are usually divided into two major subsets that are functionally and phenotypically different. The T-helper subset, also called the CD4+T cell, is a pertinent coordinator of immune regulation. Its main function is to augment or potentiate immune responses by the secretion of specialized factors that activate other white blood cells. Another important type of T cell is called the T killer/suppressor subset or CD8+T cell. These cells are important in directly killing certain tumor cells, viral infected cells and sometimes parasites. NK cells: They function as affector cells that directly kill certain tumors such as melanomas, lymphomas and viral-infected cells, most notably herpes virus and cytomegalovirus-infected cells. B-cells: The major function of B lymphocytes is the production of antibodies in response to foreign proteins of bacteria, viruses and tumor cells. Granulocytes or Polymorphonuclear (PMN) Leukocytes: Granulocytes are composed of three cell types identified as neutrophils, eosinophils and basophils, based on their staining with certain dyes. These cells are predominantly important in the removal of bacteria and parasites from the body. They engulf these foreign bodies and degrade them using their powerful enzymes.

Macrophages: They are important in the regulation of immune responses. They are often referred to as scavengers or antigen presenting cells (APC) because they pick up and ingest foreign materials and present these antigens to other cells of immune system such as T cells and B cells.

Dendritic cells: Dendritic cells, which also originate in the bone marrow, function as antigen presenting cells (APC). It is believed that they capture antigen or bring it to the lymphoid organs where an immune response is initiated.

1.2 NK Cells

1.2.1 Definition and characteristics of NK cells

Natural killer (NK) cells constitutes one subset of lymphocytes and they were first discovered in the year 1975. They are bone marrow-derived lymphocytes that were originally categorized by their large, granular morphology and their ability to lyse a variety of tumor targets and infected cells. It is a group of cytolytic lymphocytes, distinct from B-lymphocytes and T-lymphocytes that participate in both innute immunity and adaptive immunity. These cells recognize the major histocompatibility complex class I molecules on target cells, resulting in either inhibition or activation of their cytolytic potential. Natural killer cells account for up to 15% of blood lymphocytes and can be negatively defined as lymphocytes having no conventional surface antigens receptors detectable on NK cells of the myelomonocytic series (Roitt et al. 1993).

1.2.2 Function of NK cells

NK cells are crucial as a first line of defense in the body. As it was mentioned before they were first discovered by their ability to kill certain tumor lines without prior sensitization, but they can also recognize and destroy virally infected cells. In addition, NK cells can secrete significant levels of cytokines (especially IFN-γ and TNF-α) and chemokines (MIP-1 family members and RANTES). In contrast to T and B cell responses to antigen, which typically require a proliferation phase, the innate NK cell response is immediate, implying that NK cells are involved in curbing pathogens during the initial several days of infection. Indeed, there is strong evidence that NK cells contribute to the defense against intracellular bacteria and parasites and that they are critical for controlling several types of viral infection. NK cells are designed to perform the killing of certain mutant cells or virus-infected cells in one of two ways:

1.They kill cells to which antibody molecules have attached through a process called antibody-dependent cellular cytotoxicity (ADCC).

2.They are able to kill cells lacking MHC-class I molecules on their surface in a process named natural killing.

1.2.3 ADCC killing process:

The Fab portion of the antibody, binds to epitopes on the "foreign" cell. The NK cell then, binds, to the Fc portion of the antibody through its characteristic Fc receptor, CD16. The NK cell is then able to to contact the cell and release pore-forming proteins called perforins, proteolytic enzymes called granzymes and chemokines. Granzymes pass through the pores and activate a group of protease enzymes called caspases that lead to apoptosis, a programmed suicide of the infected cells. Caspases, destroy the protein structural scaffolding of the cell, degrade the cell's nucleoprotein and activate enzymes that degrade the cell's DNA. As a result, the infected cell breaks into membrane surrounding fragments, that are subsequently removed by phagocytes. ADCC killing process is also shown by the figures 1, 2 and 3 below:

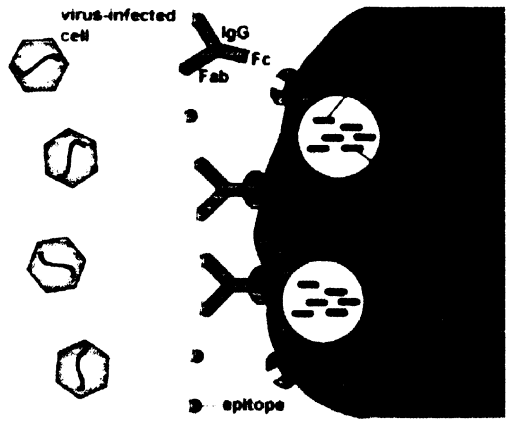

Figure 1 First stage of ADCC, recognition of antibody coated target cell through Fc receptors on NK cells

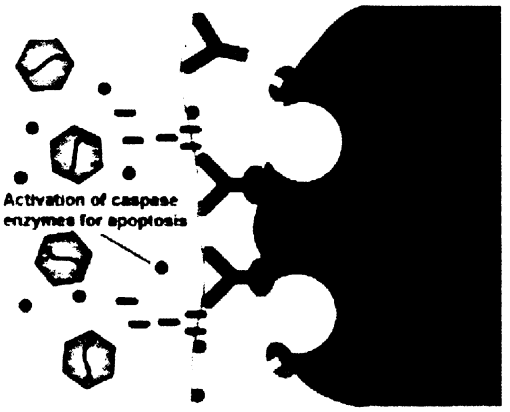

Figure 2 Second stage of ADCC, delivery of granzymes into a performing permeabilized target cell

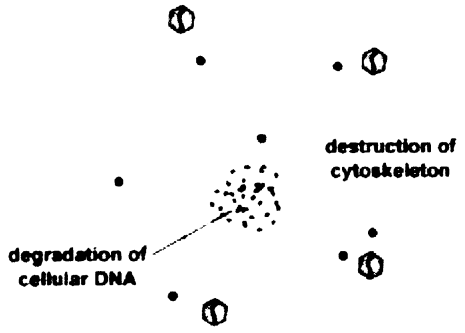

Figure 3 Residual bodies formed from the lysed target cell

1.2.4 Natural killing process

NK cells appear to use a dual receptor system in determining whether to kill or not to kill human cells. When cells are either under stress, or are infected, various stress-induced molecules are produced and are put on the surface of that cell. The first NK cell receptor, called the killer-activating receptor, recognizes these stress-induced molecules. This interaction, sends a positive signal which it has bound, unless the second receptor cancels that signal. This second receptor, called the killer-inhibitory receptor, recognizes MHC class I that are also usually present on all nucleated human cells. If MHC class I are expressed on the cell, the killer-inhibitory receptors send a negative signal that overrides the activation signal and prevents the NK cell from killing that cell. The above process is shown in the figures 4 and 5 below:

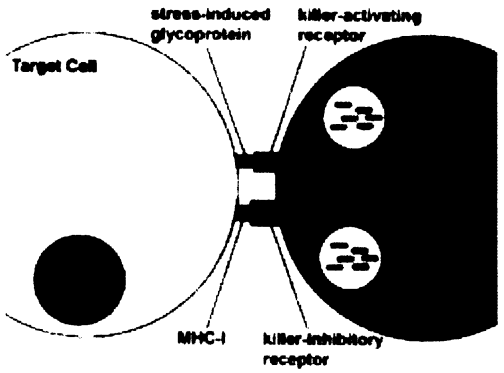

Figure 4 Inhibition of killing of MHC class I positive target cell

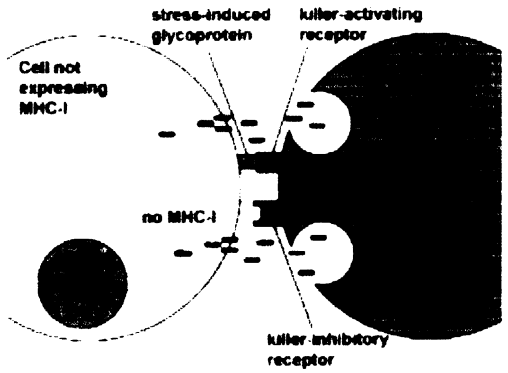

Figure 5 Killing of target cell lacking MHC class I induced by recognition of the stressed-induced protein

Despite the well-described antitumor activity of NK cells in vitro, and in certain vivo models, their role in the defense against spontaneous neoplastic transformation, remains yet to be established.

1.3 Lectins and their role in the immune system

1.3.1 Brief information about the history of lectins

According to an old definition "Lectins are multivalent carbohydrate-binding proteins or glycoproteins except for enzymes and antibodies". Such a narrow definition, however, seems no longer relevant, because a significant number of exceptions are evident now. For today, a more flexible interpetation would be accepted, e.g. "lectins or phytohemagglutinins", are proteins of nonenzymatic, nonimmune origin that bind carbohydrates reversibly without inducing any change in the carbohydrate bound.

Lectins were first described in 1888 by Stillmark working with castor bean extracts. Many members of the lectin family agglutinate (clump together) red blood cells. Lectins are found in a variety of species from plants to insects to man. They serve many different biological functions, from the regulation of cell adhesion to glycoprotein synthesis and the control of protein levels in the blood (Gabius, 1997). Lectins are also known to play important roles in the immune system by recognizing carbohydrates that are found exclusively on pathogens, or that are inaccessible on host cells.

1.3.2 Structure of Lectins

One major property of lectins is their specific saccharide-binding sites. Some lectins are composed of subunits with different binding sites. These include the lectin from the red kidney bean, Phaseolus vulgaris. It is composed of two different subunits combined into

five different forms of noncovalently bound tetramers. Since subunits have very different specificities for cell surface receptors, each combination is considered to have different function. The specificity of the binding sites of the lectins suggests that there are endogenous saccharide receptors in the tissues from which they are derived or on other cels or glycoconjugates with which the lectin is specialized to interact.

1.3.3 Types of Lectins

We can separate lectin to six big families (Drickamer, 1993)

1. C-type lectins
2. I-type lectins
3. Pentraxis
4. P-type lectins
5. L-type lectins
6. Galectins

1.3.4 C-type Lectins

C-type lectins are calcium-dependent animal lectins that are carbohydrate-binding proteins of animal origin. Carbohydrate binding activity of C-type lectins is based on the function of the carbohydrate recognition domain (CRD) the structure of which is conserved among this family (Bezouška, 2004). Calcium is not only directly involved in the carbohydrate binding, but contributes to the structural maintenance of the lectin domain that is essential for the lectin activity. The C-type CRDs are incorporated in a variety of contexts of molecular organization. This fact may reflect the importance of carbohydrate recognition in diverse biological functions.

Figure 6 A representation of a C-type trimer depicts a cluster of three CRDs bound to mannose, shown as a red stick model. The adjacent neck region of α-helices is also evident. The green spheres represent bound calcium

There are seven families of C-type lectins:

1. Proteoglycans
2. Transmembrane II type receptors
3. Collectins
4. Selectins
5. Lymfocyte membranes antigens II types
6. Mannose receptors on macrophages
7. Free saccharide – bondy domains

1.4 Cell Receptors

Receptors are molecules found on the surface of every cell. With their help, the cell communicates with its surroundings. Receptors provide the cell with vital information about the state of their surrounding environment, or, on the other side, using the receptors a cell can provide information to other cells. This ability first of all important for the cells of the immune system because their job is to recognize the organism's own cells from other heterogeneous cells and react against them accordingly.

1.4.1 Lymphocyte Receptors

All kinds of lymphocytes bare various molecules on their exterior surface (besides the ones that are common for other cells too). These molecules are specific for these cells and play a key role in their function. The T-lymphocytes (or T-cells), for example, have the TCR (T-cell Receptors) and the B-Lymphocytes have the BCR (B-Cell Receptors=Immunoglobulin). When the NK-cells were discovered, immunologists were trying to isolate and identify the NK-Cell's receptor. This research was very complicated and even though many adhesive molecules and important receptors were identified, none of them was 100% specific for the NK-Cells. All these molecules were found to be for other kinds of leucocytes too.

1.4.2 Lectine Receptors on NK-cells

After long studies and extensive research on the identifying the NK receptors, a special class of receptors was discovered on their surface that were identified as molecules belonging to the 5^{th} group of the animal C-Type lectines. These receptors are coded by the "NK Gene Complex" (NKC) (Yokoyama et al. 1991) and have very significant role on the NK-cell's function because they are responsible for the cell's activation or inhibition. The NKC is located on the 6^{th} Rat chromosome and on the 12^{th} human chromosome

(Yokoyama, 1993). Another important fact is that close to these genes lies a number of other genes whose products influence the NK-Cell's functions (in one way or another).

1.4.2.1 CD69

The human activation antigen CD69 is a member of the C-type animal lectin super family that functions as a signal-transmitting receptor. It is present at the cell surface as a disulfide-linked homodimer, with subunits of 28 and 32 KDa resulting from the differential glycosylation at a single extracellular N-linked glycosylation site. Contrary to other NKC gene (natural killer cluster) products, whose expression is restricted to NK-cells, CD69 has been found on the surface of most hematopoietic lineages. It is one of the earliest markers induced upon activation in T and B lymphocytes, NK cells, macrophages neutrophils and eosinophils. It has been demonstrated that CD69 acts as a receptor of transmitting signals. Its cytoplasmic portion is constitutively phosphorylated on serine residues. Even when the actual ligand that triggers this receptor is not known, cross-linking of CD69 by specific antibodies activates the extra cellular, signal regulated kinase pathway and has been shown to induce the rise in intracellular calcium concentration, synthesis of different cytokines and/or proliferation and target lysis in interleukin-2-activated NK-Cells. In summary, CD69 wide distribution, along with its activating signal-transducing properties, suggest an important role of this receptor in the physiology of leukocyte activation.

1.4.2.2 CD94

The expression of this antigen is limited exclusively to the NK cells and a minor population of T-Cells. The CD94 receptor is a disulfide bonded dimer (SS-bond), which is described as a membrane protein type II, belonging to the family of lectins (C-type). By analyzing it's cytoplasmatic sequence it was discovered that this part of the molecule consists out of only 7 amino acids which lead to the assumption that it must be associated with some other receptor capable of transferring extracellular signals into the cell (Brooks et al. 1997). Studies conducted on the function of the CD94 receptor showed that anti-CD 94 monoclonal antibodies can have an enhancing effect, inhibitory or even absolutely no effect on the cytotoxicity of the NK cells (Carreto et al. 1997). Another important fact is that the CD94 together with NKG2 molecules creates disulfide bonded heterodimers.. This fact implies that various signals transported with the help of the CD94 receptors are mediated by a big variety of molecules from the NKG2 receptors family

1.4.2.3 Ly-49

Ly-49 family members are expressed on subsets of NK and NKT cells as disulfide-linked dimmers. The Ly-49 family belongs to a group of type II integral membrane protein that contains external domains homologous to the super family of Ca^{2+}-dependent lectins. In the cytoplasmatic domains, they contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) that engages the intracellular tyrosine phosphatases, SHP-1 or SHP-2. However, not all Ly-49 molecules contain an ITIM. Some Ly-49 family members do not display ITIMs and instead activate NK cells. Ly-49 genes are highly polymorphic and located within the NK complex, a stretch of 2Mb on mouse chromosome 6 as a complex multigene family. The nature of the T-cell receptor on the NKT cell crucially determines the profile of expressed Ly-49 isoforms. Investigations have shown that MHC class I ligands efficiently modulate the expression levels of the inhibitory receptors, and the frequencies of cells positive for the Ly-49 members.

1.4.2.4 NKG2

NKG2 is a small family of type II transmembrane proteins possessing extracellular C-type lectin domain expressed primarily on NK cells. The function of these proteins is unknown. Molecules coded with these genes were named NKG2-A to E (Gabius, 1997). These molecules have big differences in the extra cellular portion and in the intracellular portion that shows possible ligand heterogenity and passed signals.

1.4.2.5 The discovery of NKR-P1 molecule and its functions

The NKR-P1 molecule was discovered using mouse monoclonal antibodies that were reacting with certain structures located on the NK-cells of a rat. Precipitation with the antibody showed that this structure is a disulfide bonded homodimer with a relative molecular mass of 60,000 (Ryan et al. 1992). Experiments showed that it is a transmembrane type II protein containing a homologous with the lectin C-Type domains. During the search for similar molecules it was discovered that mice and rats have genes isoforms .They were later called NKR-P1 A, B and C. Human NK cells have only NKR-P1 A receptors which shows a 46% homology with the molecules found in rats and mice. The human molecule is not present on all NK-cells, though. Furthermore, NKR-P1 is present on some human T-Cells as well. Experiments proved that the NKR-P1 is an activating receptor and a very important factor in the NK-Cell's "Natural Killing" ability, which explains the fact that its expression is much higher on activated NK-Cells.

1.5 Introduction to Protein Labeling

Protein can be covalently modified in many ways to suit the purpose of a particular assay. A wide variety of immunological and other protein methodologies involve the use of labeled antibodies or other proteins. Enzymes, biotin, fluorophores and radioactive isotopes are commonly used in biotechnology applications to provide a detection signal. They can be conjugated to antibodies, avidin, streptavidin, Fc binding proteins such as Protein A or G, or other proteins. The labeled molecule can then be used in a variety of detection systems.

Most protein labeling methods involve one of four common target strategies. The most common target for chemical groups labeling is primary amines, which are found primarily on lysine residues. They are abundant, widely distributed and easily modified because of their reactivity and their location on the surface of proteins. The second most common target is sulfhydryls, which exist in proteins in reducing conditions but more often are present in oxidized form as disulfide bonds. Because sulfhydryls are less abundant than primary amines, targeting them results in more specific conjugates. If sulfhydryls are not available for labeling, they may be introduced into a protein by reduction of disulfides, chemical modification of primary amines or point mutation to introduce cysteine residues. Two other common targets are carboxyls and carbohydrates. Carboxyls, like primary amines, are abundant and easily accessible. However, they do not react as readily as amines. Carbohydrate moieties are present on glycoproteins. If the glycosylation sites are not integral to a given protein function, they can often be modified without significantly altering protein activity. Labeling carbohydrates is a two-step process because the carbohydrates must first be oxidized to create reactive aldehydes.

1.5.1 Fluorescent Probes

Antibody molecules can be labeled with any of a number of different fluorescent probes currently available from commercial sources. Each probe option has its own characteristic spectral signals of excitation (or absorption) and emission (or fluorescence).Many derivatives of these fluorescent probes possess reactive functional groups convenient for covalently linking to antibodies and other molecules. Figure 7 shows the reaction of fluorescein isothiocyanate (FITC), one of the most common fluorescent probes, with an antibody molecule (Invitrogen, 2005).

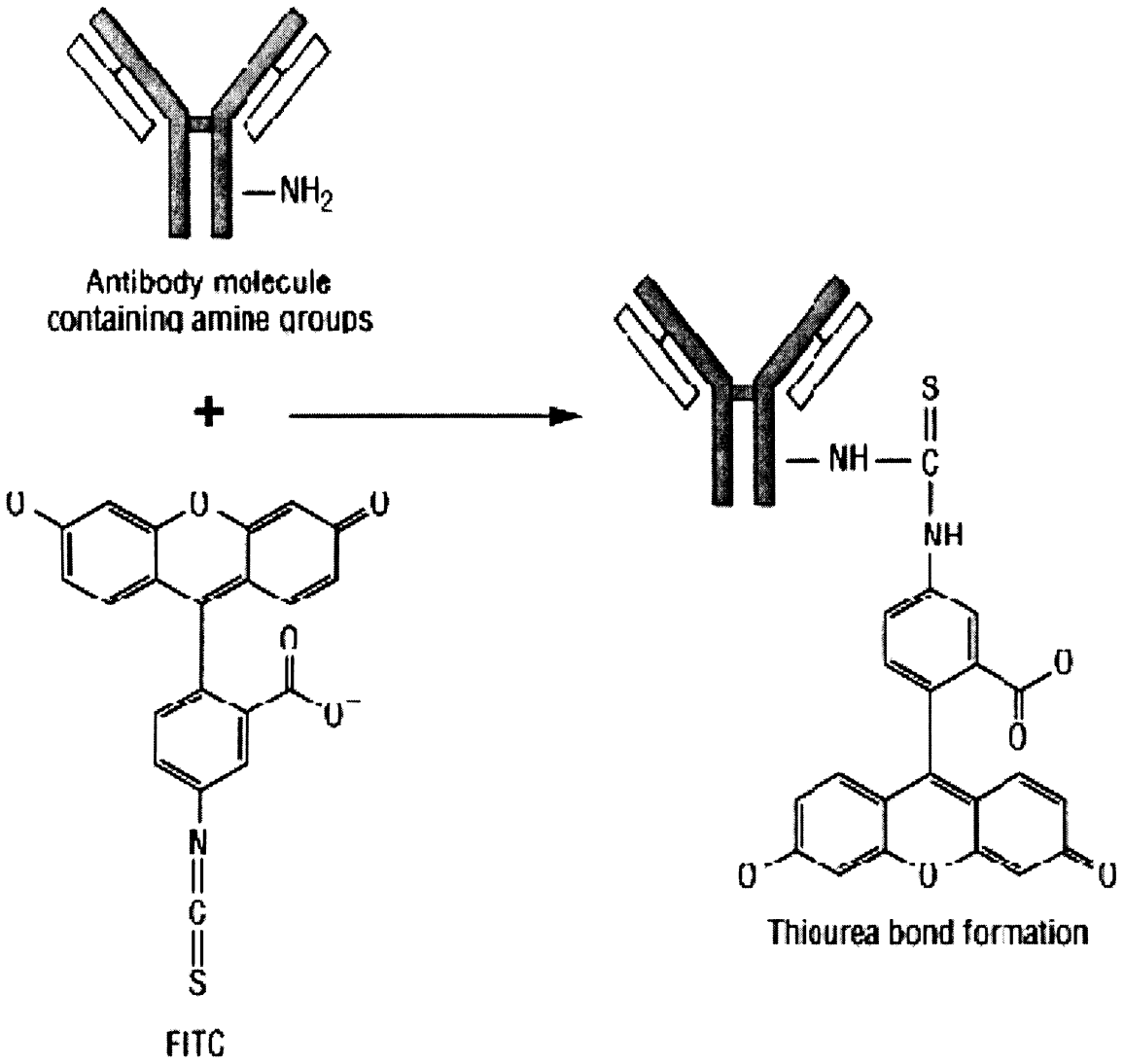

Figure 7 FITC reaction with a primary amine

1.6 Glycomics (Raman et al. 2005)

Glycome is the set of sugars that organism or cell makes. These sugars play a vital role in making the cell to work as do the proteins. They combine to form giant molecules such as carbohydrates and cellulose.

Carbohydrates (glycans) cover the surface of most (if not all) living cells and organisms in the form of diverse glycoconjugates. Glycans create a landscape of recognition sites, barriers, and carriers that help control the rythms of metabolism from conception to catabolism. Generally, glycans represent the first and crucial interface to the cell's biotic and abiotic environment, mediating recognition and communication processes. Thus, they control immunological recognition, cell-cell adhesion, pathogen attack and protein folding and placement.

Glycans are generally assembled of diverse monosaccharide building blocks that are glycosidically linked to each other at different positions. Consequently, glycans show a high structural diversity reflecting in general functional diversity. Also, it is a multidisciplinary challenge to decode the information content displayed by glycan structures in various biological contexts.

2 Aim of the work

NKR-P1 molecule is one of the most important receptors of NK cells. This is, one of the main reasons, why, their study can give us several key information in today's immunology's activities. Aim of this work, was the optimalization of fluorescent labeling protocols for NKR-P1A and B proteins, with the use of Fluorescein isothiocyanate (FITC), [5-(and 6)-carboxyfluorescein succinimidyl ester] (NHS-Fluorescein) and [5-(and 6)-carboxytetramethyl rhodamine succinimidyl ester] (NHS-Rhodamine). Furthermore, our work was to determine the biological activities of recombinant human NKR-P1 protein by using carbohydrate arrays.

3 Experimental Procedures

3.1 Materials

Proteins:

- *NKR-P1A, NKR-P1B were provided by my supervisor doc.Bezouška.* They are highly purified proteins obtained from bacterial inclusion bodies after *in vitro* refolding and purification by three chromatographical steps.

Other chemicals:

• Acrylamide	Sigma,USA
• Sodium azide	Sigma,USA
• DTT	Jersey Lab Supply,USA
• Tris	Jersey Lab Supply,USA
• SDS	Jersey Lab Supply,USA
• TEMED	Serva,SRN
• FITC	Sigma,USA
• DMSO	Sigma,USA
• Methanol	Lach-Ner,Czech Republic
• 2-propanol	Lach-Ner,Czech Republic
• acetonitrile	Merck,Germany
• NHS-Fluorescein	Sigma,USA
• NHS-Rhodamine	Sigma,USA
• BSA	Sigma,USA
• Ammonium persulfate	Sigma,USA
• CBB	Serva,USA
• Ethanol	Sigma,USA

Buffers:

- PIPES: 10Mm PIPES pH 6,8 , 49mM NaCl, 1mM NaN_3
- PBS: 120 mM NaCl 27 mM KCl, 10mM NaH_2PO_4, 1mM NaN_3, pH=7,5
- Tris: 20mM TRIS ,1mM NaN_3, pH=8
- 1xSDS-FAGE Running buffer
- 2xSDS-FAGE Running buffer

3.2 Used apparatus

• automatic pipette (10,20,200,1000 µl)	Gilson,USA
• magnetic stirrer MM2A	Laboratorní přístroje,CZ
• Analytic balance AND ER 60A	AND,USA
• pH metr	Beckman,USA
• Apparatus for gel electrophoresis	Sigma,USA
• Centrifuge J2-21	Beckman,USA
• Small laboratory centrifuge	Hermle, Germany
• Shaker	Thermolyna,USA
• Plate reader Safire^2	Tecan, Austria
• Protein purification system BioSys510	Beckman, USA

3.3 Methods

3.3.1 Protein determination

Protein in NKR-P1 preparation was determined using Bradford assay (Bradford, 1976) performed on microtiter plates in a total volume of 200 µl. BSA was used for the calibration in the concentration range 0.1 mg/ml to 0.5 mg/ml. The PIPES buffer was used as blank. The absorbance readings were recorded using Safire^2 plate reader.

3.3.2 SDS electrophoresis and PVDF blot

NKR-P1 proteins were analyzed by SDS polyacrylamide gel electrophoresis using a buffer system suggested by Laemmli (Laemmli, 1970). After the end of the electrophoresis separation, the gel was cut into halves, and one half was electroblotted onto PVDF membrane (Sambrook and Russell, 2001). Separated proteins were stained with Coomassie Brilliant Blue R-250, and destined in a destaining solution containing 35 % ethanol and 10 % acetic acid. Gels were stored in 10 % acetic acid.

3.3.3 N-terminal sequencing

N-terminal sequencing was performed using automated Edman degradations on Procise 491 instrument (Applied Biosystems, Foster City, CA, USA) using the Pulsed liquid method provided by instrument manufacturer. PTH amino acids were analyzed *on line*, and the evaluation of the sequence was done employing SequencePro 2.1 software.

3.3.4 Protein labeling protocols

NKR-P1 proteins were labeled with the use of three reagents: FITC, NHS-Fluorescein and NHS-Rhodamine .

For FITC labeling, 5 mg of the reagent was dissolved in 100 µl of organic solvent, and 10 µl of this solution was mixed with 40 µl of PBS containing 50 µg of the proteins. The mixture was incubated for two hours at 37°C with occasional gentle agitations. Thereafter, the mixture was dialysed overnight against PBS buffer, and the residual FITC was separated from the protein by gel filtration.

For fluorescein-NHS and rhodamin-NHS labeling, 1 mg of the reagent was dissolved in a mixture of methanol and DMSO (50:50, by volume), and 10 µl of this solution was added to the protein in PIPES buffer, and incubated as above. Thereafter, the uncoupled reagent was separated form the labeled protein using a Centricon 10 device.

3.3.5 Analysis of the labeled proteins

The labeled proteins were analyzed by SDS electrophoresis and N-terminal sequencing as described above. Moreover, the visible spectra of both the reagents and the labeled proteins were recorded at 300 – 700 nm using the absorbance function on the Safire^2 plate reader. Finally, the fluorescence emission spectra were recorded for all the labeled proteins using

the parameters given below. For fluorescein measurements, we used bottom fluorescence, excitation wavelength 496 nm, emission wavelength 519 nm, excitation bandwith 5 nm, emission bandwith 20 nm, gain 50. For rhodamine measurements, we used bottom fluorescence, excitation wavelength 546 nm, emission wavelength 577 nm, excitation bandwith 5 nm, emission bandwith 20 nm, gain 80.

3.3.6 Binding experiment with the labeled proteins

Microplate wells were coated with the indicated carbohydrates in the form of BSA conjugates or dendrimers (10 μg/ml) using PBS as the binding buffer by an overnight incubation. The next day the ligand was aspirated, and the wells were blocked in 2 % BSA in PBS for 2 h at 4 °C. Blocked wells were overlayed with an increasing concentration of the labeled proteins using the threefold serial dilutions. After another 2 h in the cold room, plates were washed three times with PBS, overlayed with another 100 μl of PBS, and the fluorescence was measured as above. Specific binding was calculated by substracting the fluorescence in wells without the ligand.

4 Results and discussion

4.1 Analysis of the starting NKR-P1 preparations

Before the labeling experiments it was necessary to verify the quality of the starting NKR-P1 preparations including the availability of the ε-amino groups of lysine residues used for the introduction of the label. The initial analysis was performed by SDS electrophoresis of 10 μg of the starting protein preparations on 15 % polyacrylamide gels, that confirmed the size and intactness of the protein preparations (Fig. 8). The separated proteins were also electroblotted, and subjected to automated Edman degradation in the protein sequencer. This analysis served two purposes: first, it was aimed at the final verification of the identity of the isolated NKR-P1 receptors, and second, it was aimed at the verification of the presence of the free ε-amino group on the lysine residues (approx. 10 residues per protein molecule) in the protein moiety. Unfortunately, due to technical problems with the

PROCISE protein sequencer, only a limited number of N-terminal amino acids could be called. For NKR-P1A protein, the sequence was called as MENLSKTGS (9 amino acid residues) which was in a complete agreement with the expected sequence for this protein. Moreover, the 6^{th} amino acid turned out to be a lysine residue which, according to its mobility on the reverse phase chromatograms, indeed contained a free intact e-amino group. In the case of NKR-P1B protein, only 5 N-terminal amino acid residues could be called. These were MENRT, again in an absolute agreement with the expected amino acid sequence.

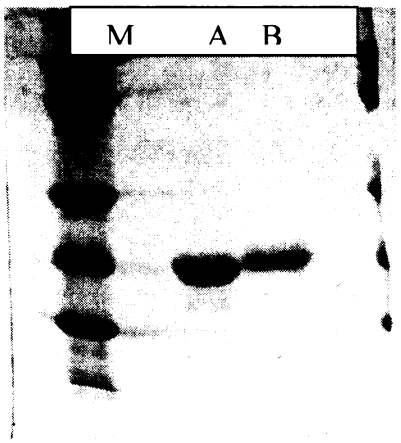

Fig. 8. Analysis of the starting preparations of NKR-P1A and NKR-P1B by SDS electrophoresis. While both proteins migrate near the 20 kDa marker, the apparent size of NKR-P1B (lane B) is somewhat larger compared to NKR-P1A (lane A). Molecular weight markers were (from the top) BSA (65 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya trypsin inhibitor (20 kDa), and lysozyme (14 kDa).

4.2 Labeling of NKR-P1 using isothiocyanate chemistry

The first approach used for the introduction of protein label into NKR-P1 was based on the use of isothiocyanate chemistry, because it is well established for the labeling of proteins such as antibodies or lectins, and the reagents such as FITC are easily available. However, FITC is not soluble in aqueous solvents, and has to be solubilized in organic solvents. Experiments performed using methanol, ethanol, 2-propanol and acetonitrile revealed a good compatibility of the protein with these solvents. However, methanol provided the highest degree of protein labeling. On other hand, other solvents such as dimethylsulfoxide

and dimethylformamide were not suitable because they caused the precipitation of the labeled protein. The overall degree of modification using FITC was very low (especially for NKR-P1B), and led us to look for additional reagents.

4.3 Labeling of NKR-P1 using N-hydroxysuccinimide chemistry

Proteins labeled with the use of *N*-hydroxysuccinimide chemistry were first characterized by absorption spectroscopy in the 300 – 700 nm range of the spectrum, and the spectra of the labeled proteins were compared with those of the activated fluorophores. A notable shift in the absorption maxima could be observed for both reagents: while the maximum of the free NHS-fluorescein was at about 496 nm, this maximum shifted to 466 nm after the reaction with the protein (Fig. 10). Similarly, when rhodamine reacted with the protein, its absorption maximum shifted from 546 nm to 516 nm (Fig.9). Moreover, the above spectral characterization allowed us to calculate the degree of substitution of the protein by the label. It was calculated from the amino acid sequences of both NKR-P1 proteins (Plihal et al. 2004) that there were about 35.2 nmols of lysine amino groups (the primary target for the labeling reaction) in the labeled protein. Of these, approximately 19.5 nmols was modified by NHS-fluorescein, and approximately 27.5 nmol by NHS-rhodamine. From these data, we could calculate the degree of modification by fluorescein as 55 %, and that by rhodamine as 78 %. This degree of modification was very high considering that some lysine amino groups may be buried in the structure of the protein, and thus may not be available for the modification reaction. Furthermore, the modification using NHS reagents was much higher than that achieved by FITC, and this labeling procedure is thus preferred.

The labeled proteins were also characterized by the measurement of the fluorescence emission spectra in order to find the characteristics suitable for their subsequent detection. We used the standard excitation maxima, and found that the standard emission maxima for fluorescein and rhodamine, respectively, can be found for the measurements of the labeled proteins. Interestingly, there has been very little

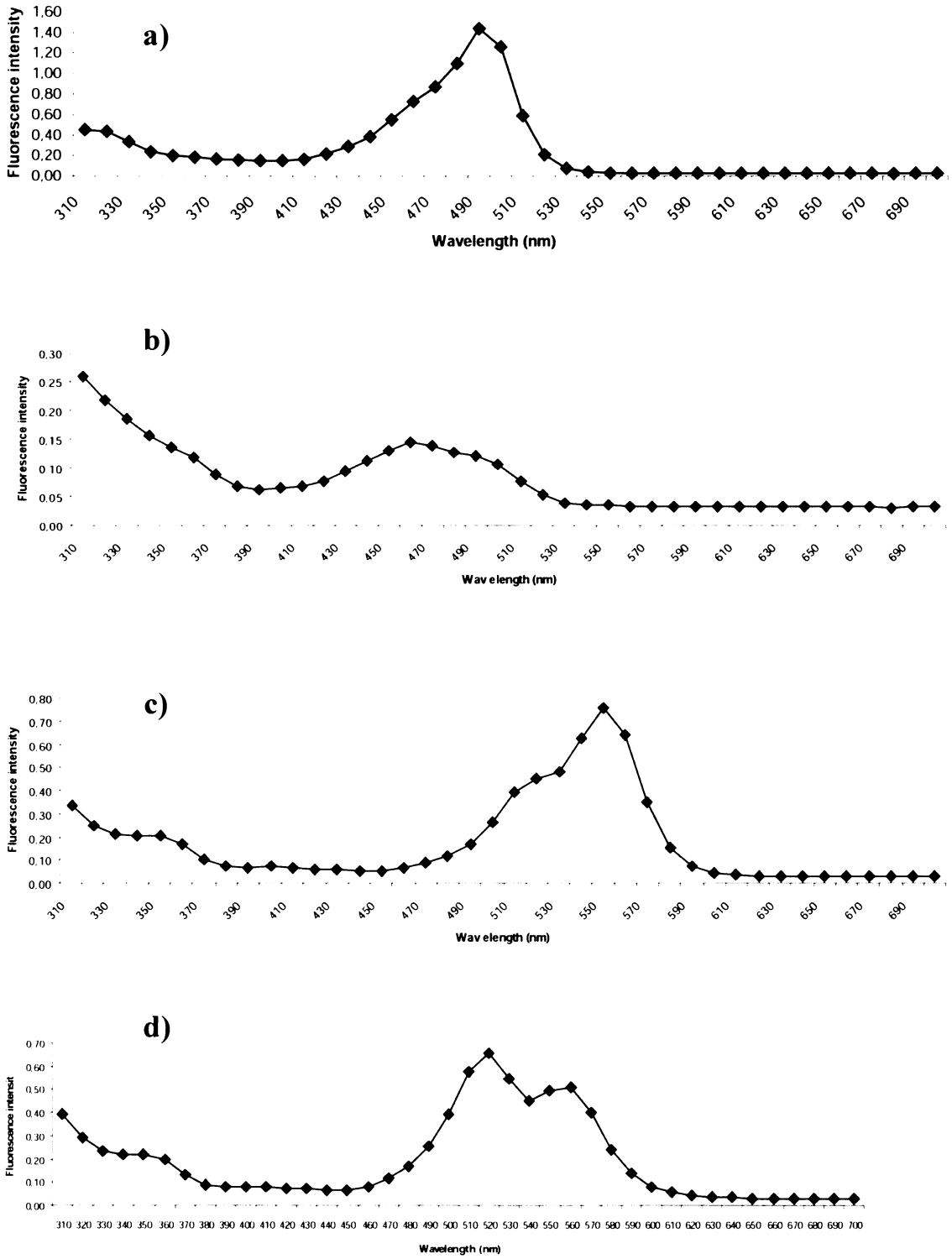

Fig. 9 The comparison of the absorption spectra of the activated NHS fluorescent labels with those of the labeled proteins. Spectra of NHS-fluorescein (a), fluorescein-labeled NKR-P1A (b), NHS-rhodamine (c), and rhodamine-labeled NKR-P1A protein.

interference between the fluorescein and rhodamine measurements, which would allow to use our labeling protocol for the simultaneous measurements of binding of NKR-P1 isoforms (fluorescein-NKR-P1A and rhodamine-NKR-P1B) to the carbohydrate arrays when using the modern glycomic technologies.

In order to further evaluate the labeling of both proteins, the labeled preparations were analyzed by SDS electrophoresis and N-terminal sequencing. The results shown in

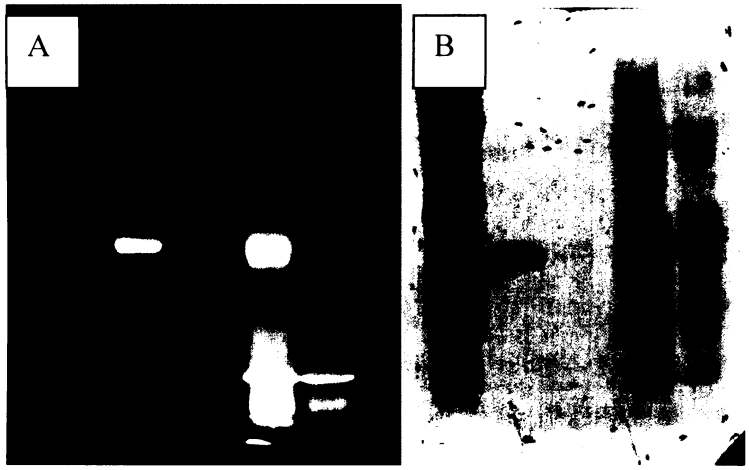

Fig.10 Analysis of the labeled proteins by SDS electrophoresis with fluorescent detection (A), and PVDF blot of the separated proteins after staining with Coomassie Brilliant Blue (B).

Fig.10 clearly indicate that the major form of the labeled protein corresponded to the monomeric subunit of both NKR-P1A and NKR-P1B. The shift in the molecular mass corresponded well to the measured degree of modification (about 5 kDa corresponding to approximately 10 molecules of the label attached to one molecule of the protein).

In order to further verify the course of the labeling reaction, the proteins were subjected to 10 cycles of N-terminal sequencing, which provided the sequence MENLSXTGSP and MENRTXTTDS for NKR-P1A and NKR-P1B, respectively. In both instances there were empty cycles (X) at the expected positions of the lysines, indicating that these residues were modified during the labeling reaction.

4.4 Binding activities of the labeled proteins

Because of the extensive degree of labeling of the receptors by both NHS reagents it was necessary to verify the biological activities of these proteins, and to check, that these activities were not impaired by the labeling reactions. Therefore, we used the standard plate binding assays to look at the carbohydrate binding activities of the labeled receptors. The results of this experiment are documented in Fig. 11. The results obtained for fluorescein labeled and rhodamine labeled proteins were very similar, once again proving the versatility of the used labels and procedures. The binding of the labeled proteins to the well known negative saccharides such as D-mannose was very little indicating that the labeling process did not damage the structure of the protein, and did not cause any nonspecific binding. On the other hand, the binding to the positive ligands such as N-acetyl-D-glycosamine was proved to be saturable. Furthermore, the labeled proteins retained the specificity for complex oligosaccharide ligands, such as the polyamidoamine dendrimers (GlcNAcP) that are specific for NKR-P1A (Plihal et al. 2004) and comb-like dendrimers known to bind NKR-P1B (Fig.11).

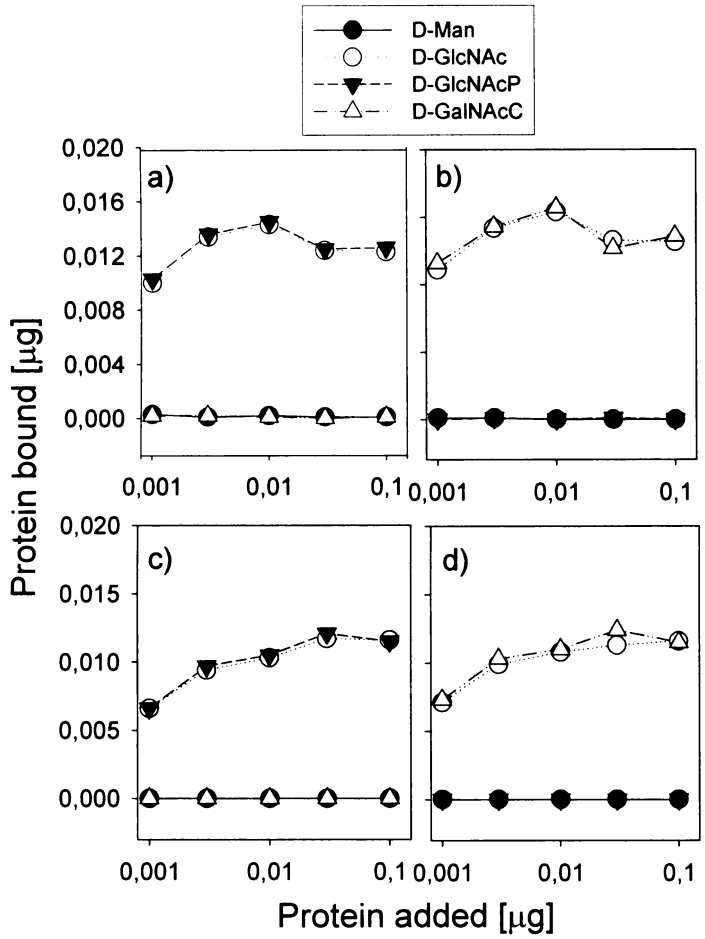

Fig. 11. Binding of fluorescein labeled NKR-P1A and NKR-P1B (panels a) and b), respectively) and of rhodamine labeled proteins (panels c) and d)) to a series of carbohydrates immobilized onto plastic wells. The binding is expressed as µg protein bound after the subtraction of nonspecific binding to wells without any ligand.

5 Conclusions

- FITC in methanol is suitable for the labeling of NKR-P1A protein but the yields and degrees of modification using this reagent were not optimal
- In order to avoid the damage of the labeled proteins, mild reagents based on the NHS chemistry were selected
- The use of the latter chemistry resulted in a high degree of modification, high specificity of the detection of the two isoforms while fully retaining the biological activity and carbohydrate specificity of the corresponding receptors
- **The methodology that we developed proved successful for the preparation of highly active lectins and is thus suitable for the prospective use in the modern carbohydrate array technologies**

References

Bezouška, K.: Collect. Czech. Chem. Commun. 69, 535-563 (2004)

Bradford, M.M.: Anal. Biochem. 72, 248-254 (1976)

Brooks, A.G., Posch, P.E., Scorzelli, C.J., Borrego, F., Coligan, J.E.: J.Exp.Med.185, 795-800 (1997)

Carreto, M.,Cantoni, C., Bellon, T., Bottino, C., Biassoni, R., Rodrígez, A., Pérez-Villar, J.J., Moretta, L., Maretta, A., López, M.: Eur. J. Immunol. 27, 563-567 (1997)

Drickamer, K.: Curr. Opin. Struct. Biol. 3, 323 (1993)

Gabius, H.J.: Eur. J. Biochem. 243, 543-576 (1997)

Invitrogen (2005) www.probes.com/handbook

Laemmli, U.K.: Nature 227, 680-685 (1970)

Plihal, O., Byrtusova, P., Pavlicek, J., Mihok, L., Ettrich, R., Man, P., Pompach, P., Havlicek, V., Husakova, L., Bezouska K.: Collect. Czech. Chem. Commun. 69, 631-644 (2004)

Raman, R., Raguram, S., Venkataraman, G., Paulson, J.C., Sasisekharan, R.: Nat. Methods. 2, 817-824 (2005)

Roitt, I.M., Brostoff, J.,David, K.: Immunology, Third Edition (1993)

Ryan, J.C., Turc, J., Niemi, E.C., Yokoyama, W.M., Seman, W.E.: J. Immunol. 149, 1631 (1992)

Sambrook, J.F., Russell, D.W.: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (2001)

Yokoyama, W.M.,Ryan, J.C., Hunter, J.J., Smith, H.R.C., Stark, M., Seaman, W.E.: J. Immunol. 147, 3229-3236 (1991)

Yokoyama, W.M.: Curr. Opin. Immunol. 5, 67 (1993)

Svoluji k zapůjčení této práce pro studijní účely a prosím, aby byla řádně vedena evidence vypůjčovatelů.

Jméno a příjmení s adresou	Číslo OP	Datum vypůjčení	Poznámka