SUMMARY AND CONCLUSION:

Costimulatory molecules play a pivotal role in shaping the immune response. Several such molecules and their pathways have been deciphered. Of these the most important pathway is the B7 (CD80 and CD86)/ICAM-1 pathway. These molecules, interleukins and a wide variety of factors are responsible for the kind of T cell response elicited.

Since the two subtypes of T cells (T_H1 and T_H2) are known to cross regulate each other, and the interleukins secreted by each group shape the immune response. Therefore, it is important to identify the factors responsible for generating the initial response.

Previous studies in our laboratory identified three T_H2 specific costimulatory molecules (B1, B2 and B3) expressed on the B cell membrane. In addition a T_H1 specific molecule from the macrophage membrane was also identified. *In vitro* assays indicated that these molecules were capable of skewing the T cell response to either T_H2 or T_H1 type, respectively. To further understand the role of any of these molecules in the immune system, it was essential to obtain a basic knowledge of their structure and sequence.

This dissertation was aimed at understanding and further characterization of the B3 molecule. The primary target was to obtain all information regarding the gene sequence and its significance with regard to the function of this molecule. LPS activated B cells and the A20 (TIB 208) cell line were utilized in this study. A20 is an ideal choice, since it has been widely used as an antigen presenting cell and therefore expresses the desired protein. This was confirmed by purifying the protein from the A20 cell membrane and an *in vitro* proliferation assay of purified CD4+ T cells.

Initial studies, yielded the surprising information that B3 shared homology to Pyruvate kinase. Pyruvate kinase is a well characterized, cytosolic enzyme, involved in the glycolytic pathway. Based on these findings, the first objective was to understand the degree of homology between the two proteins and the relevance of these similarities/ differences, taking into consideration the functional aspects.

Cloning was initiated by designing primers from the sequence information available. The PK sequence information has been published previously and the B3 sequence information was obtained from peptide sequencing. PCR amplification was done using cDNA as well as genomic DNA as template with an aim to obtain as many

B3/PK like sequences in the cell. From the initial sequencing and cloning it was inferred that 450 bp from the "C" terminal end is common to both PK and B3.

To further validate the findings, antibodies were raised against B3, PK and the expressed protein corresponding to the 450 bp region, mentioned above. Western blotting experiments reconfirmed that all three proteins have some regions that are homologous.

Armed with the data that B3 and PK share homology, both at the gene level and at the protein level, we proceeded to study the functional aspects.

PK is known to act on PEP and catalyze its conversion to Pyruvate, literature also indicates that it has atleast two additional functions. Could it be possible that a third function, that of costimulation can also be ascribed to Pyruvate Kinase? Since B3 is known to costimulate T cells, and have homology to PK, could it possess some Pyruvate Kinase - like enzymatic activity?

To answer these question we checked PK for costimulatory activity in an *in vitro* system, vice versa, we checked B3 for its ability to convert PEP to Pyruvate. Neither result was positive. In a further experimental setup, we preblocked B3 with the three antibodies mentioned previously. It was observed that α B3 as well as α 19 kD significantly blocked proliferation, however blocking with α PK was not to the same extent. The inference from this study was that although the proteins are similar, there is some basic factor responsible for the different functions.

Until this study the sequence information of the 3' region was available to us. The possibility remained that the two sequences had entirely different 5' sequences that were responsible for the observed differences. Amplification using the 5' RACE method was done to obtain this information, and the sequences of the two proteins were shown to be largely similar.

The difference in size, function and localization can be attributed to post translational modification of the protein. There exists the possibility that a glycosylated form of PK may be targetted to the cell surface and modified to a functional B3. Such a situation has been reported with the tyrosyl tRNA synthetase, a cytosolic enzyme involved in translation. Under certain conditions the enzyme is secreted to the cell surface and cleaved into two, each part taking on an entirely new function from the parent protein. The catalytic"NH_2" terminal binds to an IL-8 type A receptor and functions as an IL-8 like cytokine. An Endothelial monocyte activating polypeptide II like activity is observed with the carboxy terminal fragment. This fragment plays a major role in leukocyte and monocyte chemotaxis and

stimulates the secretion of myeloperoxidase, tissue factor and TNFα. Taking into consideration the 5' sequence information it was evident that in our case the "N" and "C" terminals are common to both PK and B3. Therefore, the possibility that PK is cleaved and part of it functions as B3, does not hold true.

The second possibility is that PK itself takes up the role of a costimulatory molecule when it is glycosylated and membrane expressed. Such a situation occurs with the glycolytic enzyme Glyceraldehyde -3 -Phosphate dehydrogenase. It can be found in a glycosylated and membrane bound form and takes on multiple roles in addition to its enzyme activity. In this case, the factors responsible for targeting to the membrane surface have not been elucidated until now. In addition to this enzyme, several other glycolytic enzymes have, in the past, been assigned multiple roles in the cell. Pyruvate Kinase itself is known to function as tubulin destabilizing agent and a cytosolic thyroid hormone binding protein.

Thirdly, since no difference is obvious in the cDNA and mRNA of these two protens, it is possible that splicing of "inteins" may occur at the posttranslational stage. This may result in a smaller protein with different functions.

These findings have raised numerous interesting possibilities that could be further exploered. The full length gene that has been cloned and sequenced requires to be expressed in a eukaryotic system. In such a system the significance of glycosylation and other posttranslational modifications on the functioning of this protein can be understood. Unpublished reports (Prof. Surolia's laboratory, IISc, Bangalore) provide information on the vital necessity for accurate glycosylation of the protein. Plant lectins cloned in both bacterial and eukaryotic expression systems failed in the functional aspects. This points to the fact that not only is glycosylation important but subtle differences in the environment or cell type may be essential for the specific glycosylation of a protein, thereby contributing to its functional properties. Therefore the choice of the expression system is vital to provide the required conditions in order to obtain an active protein. Future experiments will attempt at identifying such expression systems, where Pyruvate Kinase can undergo the glycosylation required for its role as B3. Expression of this cloned gene can be attempted in a B cell line that may be capable of providing the required posttranslational modifications.

Despite the fact that there is no obvious difference at the mRNA level, there is a vast difference between the size and functions of the two proteins. It appears that intein mediated posttranslational splicing could be the mechanism involved in

generating B3 from Pyruvate Kinase. The presence and identification of inteins in the sequence, is also another aspect that could provide information regarding these molecules. Future experiments could include identification and analysis of these sequences and studies in parallel to that of the vacuolar membrane H^+-ATPase (VMA1) protein of the yeast *Saccharomyces cerevisiae.*

To date, there are no reports of receptors for B3 or Pyruvate kinase. Such ligands could be identified on the T cell surface. Analysis of B3 expression on different cells of the immune system as well as its modulation could provide an accurate picture of the importance of this molecule in costimulation. Complete characterization of B3 and its ligand could then assist in elucidating the precise pathway and role of B3 in costimulation and activation of T cells. The role of this molecule in generating and modulating the T_H2 response in autoimmune diseases could be a further extension of this work.