Organization of complex situations in the immune system

Conference: Signal processing through protein complexes

Introduction

Over the last few decades, the search for and identification of new genes has been the main focus of molecular biology. Due to many collaborative efforts and the successes of genome sequencing projects, particularly over the last few years, most of the genes of yeast, worm, fly and man have now been catalogued. The identification of DNA encoding a particular protein, however, does not suffice to explain all aspects of its expression and function. One great complication is that the functional properties of a given protein are not solely dependent on its own activity, catalytic or otherwise, but mostly rely on the influences of other components of complexes in which they participate. These complexes can be highly ordered dynamic structures, and may serve to create, store and transduce biological information. The functional importance of protein–protein interactions has become strikingly evident, particularly in the field of signal transduction. Over the course of evolution, a wide variety of modular binding domains with specificity for distinct sequence motifs has developed to organize these interactions properly, and the identification of these domains has already created an extremely powerful paradigm for understanding signal transducing processes. The 81st International Titisee Conference organized by the Boehringer Ingelheim Fonds (<http://www.bifonds.de>), a foundation for basic research in medicine, addressed the current state of our understanding of these interactions. About 40 researchers from Europe and the USA met in Titisee, Germany from April 12–16, 2000 to share their knowledge and newest data on this interesting aspect of molecular biology. The focus was on the organization of protein complexes in cells of the immune system, with adaptor proteins being the central elements for the nucleation of these complexes, which are responsible for signal integration and propagation.

Modular binding domains

The first protein interaction domains to be described were the Src homology 2 and 3 (SH2 and SH3) domains, which were initially found in the Src tyrosine kinase (Koch *et al.*, 1991). SH2 domains bind protein regions containing phosphotyrosines (pTyr), whereas SH3 domains interact with proline-rich targets. Meanwhile, however, it is evident that more than a single modular binding domain exists for a given target class. In addition

to SH2 domains binding to pTyr residues in the context of their adjacent three C-terminal amino acids, phosphotyrosine-binding (PTB) domains have been identified that also recognize phosphorylated tyrosines. In their case, the interaction is dependent on the proximal N-terminal amino acids of the pTyr residue (van der Geer and Pawson, 1995). In keeping with the different binding specificities for residues surrounding the pTyr, the folding topology of a PTB domain is distinct from that of SH2 domains (Zhou *et al.*, 1995).

As is the case for the SH2 binding partners, SH3 domain targets can also interact with other protein domains; WW, EVH1 and GYF domains have all been found to bind to polyproline-rich proteins. Again, binding specificity is determined by protein conformation. Whereas SH3, WW and EVH1 domains bind proline-rich ligands in a left-handed, type II poly-L-proline helical conformation, the GYF domain recognizes a different conformation (Freund *et al.*, 1999). Other important modular domains are FHA, EH and PDZ. These domains recognize phosphothreonine residues, Asn-Pro-Phe (NPF) consensus motifs and C-termini of proteins, respectively (reviewed by Forman-Kay and Pawson, 1999). In addition, both PDZ and SAM domains are capable of homodimerizing. To further complicate the picture, not only do modular binding domains interact with other protein structures, but certain of them, such as the PH and FYVE-finger domains, adhere specifically to certain phospholipids.

Tony Pawson (Toronto, Canada) pointed out in his keynote lecture that evolution must have made use of combinatorial chemistry with regard to the development of modular binding domains and signalling pathways. After the first introduction of SH2 and SH3 domains, even minute changes within them could have created domains with altered binding characteristics. Pawson went on to illustrate that this holds true by describing experiments in which the Thr residue of the +3-binding pocket of the Src SH2 domain was changed to Trp, the corresponding amino acid in the SH2 domain of the adaptor protein Grb2. This resulted in a switch in the selectivity of the protein to that of the Grb2 protein. Pawson also discussed the fact that the tertiary folds of the PTB and PH domain superfamily bear a strong resemblance to the folds of EVH1 domains (reviewed by Forman-Kay and Pawson, 1999) and that it is thus possible that a common ancestral domain functioned as a regulatory binding module. This then could have diverged, yielding multiple domains with distinct binding specificities.

SLP family proteins and other cytosolic adaptors

Fig. 1. Schematic representation of the proteins of the SLP family. The adaptor proteins SLP-76, SLP-65 (BLNK or BASH) and Clnk share the same overall structural organization. In addition to the C-terminal SH2 domains (SH2), they contain multiple potential tyrosine phosphorylation sites (P), several proline-rich SH3 domain-binding motifs (SH3) and one or two basic regions (Lys, His, Arg). (Figure courtesy of J. Wienands, Freiburg, Germany.)

Proteins interacting with proteins interacting with proteins...

Signalling proteins can be classified roughly into two major groups based on their domain structures. The first includes proteins that possess catalytic activity and contain one or more additional modular binding domains. The second encompasses a fast growing group of proteins, so-called adaptor or scaffold proteins, which consist only of binding domains of either similar or distinct types. While these proteins do not have any catalytic activity of their own, they accomplish a variety of missions, e.g. nucleating and organizing larger signalling complexes, positioning signalling complexes in certain compartments within the cell, and bringing together effector and corresponding target proteins (reviewed by Pawson and Scott, 1997).

One important group of adaptor proteins is represented by the SLP family, which, so far, comprises three members: SLP-76, SLP-65 (BLNK or BASH) and Clnk (Figure 1). The N-termini of these proteins contain several tyrosine residues, which are putative targets for phosphorylation. While the central regions of these proteins are proline-rich and contain some SH3 domain binding motifs, their C-termini contain SH2 domains. SLP-76 is mainly expressed in T cells, macrophages, mast cells, natural killer cells and platelets. Its central role in T-cell receptor (TCR) signalling and T-cell development is illustrated by the fact that SLP-76 $-/-$ mice are devoid of peripheral T cells (Clements *et al.*, 1998). Expression of SLP-65, on the other hand, seems to be restricted to B cells and macrophages. Although its role in B-cell development is not as crucial as that of SLP-76 in T-cell development, B-cell maturation and function are disturbed at various stages in SLP-65 $-/-$ mice (Jumaa *et al.*, 1999). Less is known about Clnk. It seems, however, to be selectively expressed in cytokine-stimulated hemopoietic cells (Cao *et al.*, 1999).

SLP-76 interacts with various proteins via its protein binding modules (see Figure 1). It is evident from studies presented by A. Weiss (San Francisco, CA), that SLP-76 functionally interacts, via Gads (Grb2-related adaptor downstream of Shc), with another adaptor protein termed LAT (linker for activation of T cells). LAT, together with SLP-76, is required for full activation of the phospholipase C- γ (PLC- γ) and Ras signalling pathways after TCR triggering. In this context, the membrane-associated LAT attracts PLC- γ to the membrane, where it is phosphorylated by the tyrosine kinase Itk, which is bound to SLP-76 (Figure 2). This shows that TCR signal transduction is strictly dependent on the coordinated interplay between protein complexes organized by different adaptors.

Common themes have appeared for antigen receptor signalling in B and T cells, with the two systems engaging different signalling elements that function in similar ways. In T cells, a simplified picture (Figure 2) depicts the tyrosine kinase ZAP70 binding to phosphorylated CD3 ζ chains of the activated receptor. ZAP70 then relays the signal by phosphorylating proteins organized by SLP-76, Gads and LAT. In B cells, the tyrosine kinase Syk, a ZAP70 homologue, binds to the phosphorylated CD79a/CD79b dimer, the equivalent of the TCR ζ chains, and transduces the signal via the adaptor SLP-65. An equivalent for LAT has not yet been found in B cells.

T. Kurosaki (Moriguchi, Japan) tested this model of common themes by reconstituting the B-cell receptor (BCR) signal transduction machinery in various knock-out DT40 B-lymphocyte lines by adding in elements from T cells. Using calcium mobilization as the read-out, he was able to show that BCR signalling in Syk $-/-$ B cells was reconstituted by expression of ZAP70, indicating that ZAP70 is capable of cooperating with SLP-65. However, expression of ZAP70 and SLP-76 in Syk $-/-$ SLP-65 $-/-$ B cells did not result in BCR-induced calcium mobilization. Therefore, SLP-76 cannot compensate for SLP-65 in this system. Further studies indicated that, unlike SLP-76, SLP-65 is found in glycosphingolipid-enriched microdomains (GEMs), which are important structures of the plasma membrane for the organization of signalling elements. Consistent with this, expression of ZAP70 and a GEM-targeted SLP76 in Syk $-/-$ SLP-65 $-/-$ B cells did restore calcium mobilization after BCR engagement. Hence, targeting of the SLP component to GEMs in the plasma membrane is crucial to its function. In T cells, the adaptor LAT, which is associated with GEMs due to its palmitoylation, probably performs the role of coupling SLP-76, via Gads, to these GEMs. Consistent with this hypothesis, expression of ZAP70, SLP-76, Gads and LAT in Syk $-/-$ SLP-65 $-/-$ B cells completely reconstituted BCR-induced calcium signalling.

It is now clear that SLP-76 and SLP-65 are coupled to TCR and BCR signalling, respectively, by different regulatory events. The question remains as to whether they activate different downstream effectors. J. Wienands (Freiburg, Germany) reported on the interactions between the SH2 domains of the Tec-family kinases, Itk and Btk, and tyrosine-phosphorylated SLP-76 and SLP-65, respectively. The Tec family kinases are critical for the activation of PLC- γ and for subsequent intracellular calcium mobilization. Overexpression of SLP-65 in B cells stimulates

Fig. 2. The transmembrane adaptor LAT is a central organizer for multiple protein complexes. After the TCR-CD4 complex is triggered by peptides bound to the MHC on antigen presenting cells, several signalling pathways are activated. LAT plays an important role in organizing functionally distinct protein complexes in at least four of these pathways, as depicted in this figure. (Figure courtesy of L. Samelson, Bethesda, MD.)

increased calcium flux after BCR engagement (J. Wienands, Freiburg, Germany), and overexpression of SLP-76 in T cells resulted in enhanced activation of the calcium-dependent NFAT promoter after TCR triggering (G. Koretzky, Iowa City, IA). The ability to rescue calcium mobilization upon BCR crosslinking was lost in a SLP-65 mutant carrying six Tyr-to-Phe substitutions in its N-terminus when put into a SLP-65 $-/-$ DT40 background (T. Kurosaki, Moriguchi, Japan). Similarly, the N-terminal tyrosine residues of SLP-76 have been shown to be crucial for optimal augmentation of NFAT promoter activity (Fang *et al.*, 1996). Whereas the expression of a wild-type SLP-76 transgene in T lineage cells of SLP-76 $-/-$ mice rescued thymocyte development as well as proliferation, expression of a SLP-76 mutant carrying three Tyr-to-Phe mutations failed to do so; the numbers of CD4⁺ CD8⁺ cells was low, the differentiation marker CD25 failed to be expressed, and the cells did not proliferate (G. Koretzky). In summary, these data clearly show that, although the signalling events required for activation of SLP-76 and SLP-65 are not easily exchangeable, both adaptor proteins are critical for comparable downstream effects in T and B cells, respectively.

In his presentation, A. Veillette (Montreal, Canada) focused on Clnk, the new SLP family member. Clnk accomplishes comparable but also unique tasks. As is the case for SLP-76 and SLP-65, Clnk overexpression leads to increased activation of the

promoters for NFAT, AP-1 and IL-2. In addition, it associates with a novel protein of 92 kDa that has not yet been found to interact with SLP-76 and SLP-65. The group of A. Veillette also identified the adaptor protein, Dok-3. The Dok proteins are characterized by an N-terminal PH domain, a central PTB domain and several potential C-terminal tyrosine phosphorylation sites. Dok-1 and Dok-2 were originally isolated because of their interactions with the Ras GTPase-activating protein (RasGAP) (Yamanashi and Baltimore, 1997; Di Cristofano *et al.*, 1998). Veillette explained that Dok-3, which is abundantly expressed in B cells and macrophages, does not bind to RasGAP. However, it does interact with two inhibitory signalling molecules, Csk and SHIP, via pTyr-SH2 interactions. Overexpression of Dok-3 in a B-cell line results in inhibition of immunoreceptor-mediated NFAT activation and cytokine release, and introduction of a Dok-3 mutant with impaired ability to associate with SHIP and Csk enhances B-cell responsiveness to BCR stimulation. Dok-3, therefore, appears to be an adaptor involved in the recruitment of inhibitory molecules, and to play an important role in the negative regulation of immunoreceptor signalling.

Transmembrane adaptors

In addition to making use of various cytosolic adaptor proteins, T lymphocytes express several transmembrane adaptor proteins

(TRAPs) that recruit SH2 domain-containing intracellular molecules to the cell membrane via tyrosine-based signalling motifs. These TRAPs usually have a very short extracellular domain, a transmembrane region and an elongated tyrosine-rich cytoplasmic tail. One example is TRIM, the TCR interacting protein reported on by B. Schraven (Heidelberg, Germany). TRIM is a disulfide-linked homodimer and associates with and comodulates signalling by the TCR-CD3- ζ complex in T lymphocytes. After TCR stimulation, TRIM becomes rapidly phosphorylated on multiple tyrosines and associates with phosphoinositide 3-OH kinase (PI3K). Overexpression of TRIM results in a two-fold upregulation of TCR expression at the cell surface. In summary, these data could indicate that TRIM is responsible for both TCR expression and the propagation of TCR-mediated signals.

PAG (phosphoprotein associated with GEMs) is another example of a TRAP (V. Horejsi, Prague, Czech Republic). This ubiquitously expressed phosphoprotein of 85 kDa associates with GEMs, binds the inhibitory protein tyrosine kinase Csk and is involved in the regulation of TCR signalling. PAG contains multiple potential tyrosine phosphorylation sites and exists in a phosphorylated state in unstimulated cells. Following activation of T cells, PAG becomes rapidly dephosphorylated and dissociates from Csk. Csk, the major negative regulator of Src family kinases, is then released from the plasma membrane where its substrates reside. PAG overexpression results in downregulation of CD3- ζ tyrosine phosphorylation, Fyn autophosphorylation and NFAT induction after TCR triggering. These findings collectively suggest that, in the absence of external stimuli, the PAG-Csk complex transmits negative regulatory signals and thus may help to keep resting T cells in a quiescent state.

A third member of the TRAP group is LAT, which has already been discussed in relation to SLP-76. As reported by L. Samelson (Bethesda, MD), due to a block in thymocyte development at the CD4⁺ CD8⁺ CD25⁺ CD44⁺ stage of T-cell development, LAT ^{-/-} mice lack mature T cells, whereas the B cells develop normally. LAT was reported to be required for PI3K-independent T-cell spreading. Spreading of J.CaM2 cells, LAT-deficient human T cells, can be restored by expression of wild-type LAT, but not by a LAT mutant unable to interact with the adaptor Grb2 and with PLC- γ . These proteins are thereby implicated in the regulation of cell spreading. Furthermore, LAT deficiency in mast cells results in defects in secretion as well as cytokine production. Both of these processes are also dependent on a functionally intact PLC- γ pathway. Further along in his presentation, Samelson stressed an important feature of adaptor proteins, namely the parallel and/or consecutive involvement in the organization of multiple protein complexes that are functionally diverse. As shown in Figure 2, after TCR triggering by the MHC-antigen complex, LAT can initiate various responses in a manner dependent on its interaction partners. These responses include Rac/cdc42 activation, actin polymerization, intracellular calcium elevation, PKC activation and Ras pathway activation.

The fact that LAT performs roles in so many different pathways illustrates that identifying binding partners of adaptor proteins will only be one of many steps toward understanding signalling in the immune system. The functionality of a specific interaction can only be resolved in detail if the complete protein complex in which a specific interaction occurs is characterized. Thus, one of the challenges in investigating protein-protein interactions is

the identification and purification of larger protein complexes, and the analysis of their subcellular distribution as well as assembly and disassembly characteristics. This process is already well under way. In the post-genomic 'proteomics' era, molecular analysis of complex biological structures and processes will become increasingly dependent on fast and high throughput methods for protein separation and identification. In M. Mann's (Odense, Denmark) approach, components of a protein complex are purified via molecular interactions using an affinity-tagged member and the purified complex is then separated—usually by 1D gel electrophoresis. Silver-stained bands are excised and identified in a two-step mass spectrometric process, using matrix-assisted laser desorption/ionization (MALDI) 'mass finger printing' first and nanoelectrospray peptide sequencing as a second technique (reviewed in Pandey and Mann, 2000). Several novel components in the NF κ B and EGF receptor signalling systems have been identified by his group. R. Aebersold (Seattle, WA) addressed the question of how to accurately quantify individual proteins within complex mixtures. His novel method employing isotope-coded affinity tags (Gygi *et al.*, 1999) should provide a widely applicable means of comparing quantitatively global protein expression.

Perspectives

In conclusion, the 81st International Titisee Conference served as a perfect platform to discuss different flavours of signal transduction. Since every type of signal processing is dependent on controlled assembly of protein complexes, it seems likely that a major focus in the coming years will be the purification and analysis of larger protein complexes and the determination of their physiological functions, as well as the individual contributions of their components. Whereas the tools to analyse proteins in a high throughput fashion are already available, the development of novel methods specifically to purify large protein complexes will be a tempting challenge for protein biochemists.

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References

- Cao, M.Y., Davidson, D., Yu, J., Latour, S. and Veillette, A. (1999) Clnk, a novel SLP-76-related adaptor molecule expressed in cytokine-stimulated hemopoietic cells. *J. Exp. Med.*, **190**, 1527–1534.
- Clements, J.L., Yang, B., Ross-Barta, S.E., Eliason, S.L., Hrstka, R.F., Williamson, R.A. and Koretzky, G.A. (1998) Requirement for the leukocyte-specific adaptor protein SLP-76 for normal T cell development. *Science*, **281**, 416–419.
- Di Cristofano, A. *et al.* (1998) Molecular cloning and characterization of p56 (Dok-2) defines a new family of RasGAP-binding proteins. *J. Biol. Chem.*, **273**, 4827–4830.
- Fang, N., Motto, D.G., Ross, S.E. and Koretzky, G.A. (1996) Tyrosines 113, 128 and 145 of SLP-76 are required for optimal augmentation of NFAT promoter activity. *J. Immunol.*, **157**, 3769–3773.

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- Forman-Kay, J.D. and Pawson, T. (1999) Diversity in protein recognition by PTB domains. *Curr. Opin. Struct. Biol.*, **9**, 690–695.
- Freund, C., Dotsch, V., Nishizawa, K., Reinherz, E.L. and Wagner, G. (1999) The GYF domain is a novel structural fold that is involved in lymphoid signaling through proline-rich sequences. *Nature Struct. Biol.*, **6**, 656–660.
- Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H. and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nature Biotechnol.*, **17**, 994–999.
- Jumaa, H., Wollscheid, B., Mitterer, M., Wienands, J., Reth, M. and Nielsen, P.J. (1999) Abnormal development and function of B lymphocytes in mice deficient for the signaling adaptor protein SLP-65. *Immunity*, **11**, 547–554.
- Koch, C.A., Anderson, D., Moran, M.F., Ellis, C. and Pawson, T. (1991) SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science*, **252**, 668–674.
- Pandey, A. and Mann, M. (2000) Proteomics to study genes and genomes. *Nature*, **405**, 837–846.
- Pawson, T. and Scott, J.D. (1997) Signaling through scaffold, anchoring and adaptor proteins. *Science*, **278**, 2075–2080.
- van der Geer, P. and Pawson, T. (1995) The PTB domain: a new protein module implicated in signal transduction. *Trends Biochem. Sci.*, **20**, 277–280.
- Yamanashi, Y. and Baltimore, D. (1997) Identification of the Abl- and RasGAP-associated 62 kDa protein as a docking protein, *Dok. Cell*, **88**, 205–211.
- Zhou, M.M. *et al.* (1995) Structure and ligand recognition of the phosphotyrosine binding domain of Shc. *Nature*, **378**, 584–592.

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