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The association of the protein tyrosine kinases p56^{lck} and p60^{fyn} with the glycosyl phosphatidylinositol-anchored proteins Thy-1 and CD48 in rat thymocytes is dependent on the state of cellular activation

Cell surface glycoproteins anchored to the plasma membrane via glycosyl-phosphatidylinositol (GPI) structures, and hence having no cytoplasmic domains, can nevertheless transmit activation signals in lymphocytes. By immunoprecipitation from detergent lysates and *in vitro* immune complex kinase reactions the GPI-anchored molecules Thy-1 and CD48 are shown to be associated with multimolecular complexes of phosphoproteins including the protein tyrosine kinases p56^{lck} and p60^{fyn} in both rat and mouse thymocytes. Moreover, the kinase activity associated with Thy-1 on rat thymocytes is shown to be dependent on the activation state of the cells, with stimulation by the lectin, concanavalin A, producing a marked decrease in Thy-1-associated kinase activity. In such activated cells, there is an increased association of kinase activity with CD48, but this may be explained in terms of increased surface expression of CD48 and of increased total kinase activity. Additional phosphoproteins of 85, 36 and 32 kDa were consistently seen as components of the complexes.

1 Introduction

Thy-1 and CD48 are leukocyte surface glycoproteins of the immunoglobulin superfamily which are attached to the cell membrane by glycosyl phosphatidylinositol structures (reviewed in [1, 2]). The functions of both molecules are unclear, but Thy-1 may be involved in the adhesion of thymocytes to thymic epithelium [3], and cross-linking of Thy-1 on mouse T cells elicits a rise in intracellular calcium [4, 5], tyrosine phosphorylation of a number of intracellular proteins [6] and is co-mitogenic with phorbol esters [4]. Thy-1 is also highly expressed on neural cells and may be involved in controlling neurite outgrowth by binding a ligand on astrocytes [7]. Cross-linking of rat CD48 has been shown to induce an accessory cell-dependent suppression of T cell proliferative responses [8], and CD48 has recently been shown to be a ligand for the T cell surface protein CD2 in mouse, human and rat [9–11].

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Abbreviations: **GAP:** GTPase-activating protein **GPI:** Glycosyl phosphatidylinositol

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Cross-linking of several other glycosyl phosphatidylinositol (GPI)-anchored proteins, such as Qa-2 and Ly-6, leads to lymphocyte activation, and it is unclear how such molecules, lacking cytoplasmic domains, might transmit such signals (reviewed in [12]). However, the requirement for an intact GPI anchor has been demonstrated for mitogenic stimulation of T cells by Qa-2 and Ly-6 [13, 14], while not being necessary for the function of molecules with clear extracellular functions such as decay-accelerating factor (CD55) [15]. The recent characterization of GPI-anchored receptors whose main function seems to be to form part of a signal transduction complex, such as the ciliary neurotrophic factor receptor [16], points to an important role for GPI anchors in cell activation.

Recent reports indicate the association of human GPI-anchored molecules CD14, CD24, CD48, CD55, CD59, and murine Thy-1 and Ly-6 with the protein tyrosine kinase p56^{lck} [17], of murine Thy-1 with p60^{fyn} [18] and p53/56^{lyn} [19]. Human CD55 has been shown to be associated with both p56^{lck} and p60^{fyn} [20]. p60^{fyn} is involved in signal transduction by the T cell receptor/CD3 complex [21], and p56^{lck} with the T cell coreceptors CD4 and CD8 [22] and with the IL-2 receptor [23]. In this report we examine the kinases and their substrates present in the immunoprecipitates of Thy-1 and CD48 both from resting rat thymocytes and from those activated by the mitogenic lectin, concanavalin A.

2 Materials and methods

2.1 Cells and antibodies

Thymocytes and spleen cells were isolated from 8–10-week-old AO RT1^{u} rats or BALB/c mice raised under specific pathogen-free conditions (Sir William Dunn School of Pathology, Oxford University). Con A blasts were prepared by culturing thymocytes or spleen cells in

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RPMI 1640 (Gibco, Glasgow), 10% FCS containing 5 μg/ml Con A (Sigma Chemical Co., Poole, GB) for the times stated. The EL4 mouse thymoma cell line was maintained in RPMI, 10% FCS. Antibodies used were as follows: W3/25 (anti-rat CD4, IgG_1); OX-7 (anti-rat Thy-1.1, IgG_1); OX-20 (rat anti-mouse Igκ, IgG_1); OX-21 (anti-human C3bi, IgG_1); OX-45 (anti-rat CD48, IgG_1); OX-42 (anti-rat CD18, IgG_{2a}); OX-78 (rat anti-mouse CD48, IgG_1); 30H12 (rat anti-mouse Thy-1.2, IgG); YTS191 (rat anti-mouse CD4, IgG_{2b}); 1B3 (anti-human $p56^{lck}$, IgG [24]); PY20 (anti-phosphotyrosine, IgG, ICN); anti-*lck* 1, rabbit anti-mouse $p56^{lck}$ antiserum, gift from J. Borst, Netherlands Cancer Institute, Amsterdam, Netherlands; anti-*lck* 2, rabbit-anti mouse $p56^{lck}$ amino acids 39–64 antiserum, gift from J. B. Bolen, Bristol-Myers Squibb, Princeton, NJ; anti-GTPase-activating protein-(GAP), raised against a glutathione S-transferase fusion protein containing the SH2 and SH3 domains of GAP, gift from J. Downward, ICRF, Lincoln's Inn Fields, London; anti-PI3K, raised against a glutathione S-transferase fusion protein with the 85-kDa subunit of phosphatidylinositol 3-kinase, AMS Biotechnology, Witney, GB; rabbit anti-human $p95^{vav}$ amino acids 528–541 antiserum, AMS Biotechnology; rabbit anti-rat and rabbit anti-mouse IgG and $F(ab')_2$ FITC conjugates (Serotec Ltd., Kidlington, GB).

2.2 Immunoprecipitations and *in vitro* kinase assays

These were performed by the method of Beyers et al. [25]. Cells (10^8 or as otherwise stated) were labeled for 30 min with neat monoclonal antibody tissue culture supernatants, lysed in 1 ml 1% Brij 96 lysis buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 μM phenylmethylsulfonyl fluoride and 0.1% BSA for 30 min at 4 °C followed by brief centrifugation (12000 × g, 10 min, 4 °C) to remove insoluble material. For direct assaying of $p56^{lck}$ activity, lysis preceded labeling with 5 μl antiserum. After preclearing with OX-21 or OX-42 IgG covalently coupled to CNBr-Sepharose beads, immunoprecipitation of mouse and rabbit antibodies was done using protein A-Sepharose beads. Immunoprecipitation of rat antibodies was done using rabbit anti-rat IgG (non-cross-reacting with mouse, Serotec Ltd.) coupled to protein A-Sepharose beads. After three washes with 1 ml lysis buffer, and two in 1 ml assay buffer (0.1% Brij 96, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5), the beads were incubated for 15 min at 25 °C in 30 μl assay buffer with 10 mM $MnCl_2$, 100 μM Na_3VaO_4, 100 μM NaF, and 5 μCi[γ-^{32}P]-ATP. Following addition of 30 μl 2 × SDS sample buffer with 5% 2-mercaptoethanol, samples were briefly boiled and subjected to SDS-PAGE on 12% gels, the gels dried and autoradiographed with Kodak X-Omat film at −70 °C with intensifying screens. For reprecipitations, labeled phosphoproteins resulting from the incubation in final assay buffer were dissociated from the beads and precipitated with a second antibody, as follows. Seventy microliters assay buffer containing 2.5% SDS was added to each sample, followed by vortexing and boiling for 5 min, and addition of 900 μl assay buffer. The beads were then pelleted and the supernatants incubated with the second antibody (5 μl antiserum, 1 h) followed by protein A-Sepharose beads (1 h, 4 °C). After pelletting and washing the beads (4 × 1 ml assay buffer), 60 μl reducing SDS sample buffer was added and the samples were boiled and analyzed by SDS-PAGE and autoradiography as described above.

2.3 Flow cytometry

Cells were labeled with monoclonal antibodies as above, washed three times in ice-cold PBS containing 0.2% BSA, and incubated with FITC-conjugated $F(ab')_2$ rabbit anti-mouse or anti-rat IgG, washed and analysed using a Becton Dickinson FACScan.

3 Results

3.1 Thy-1 and CD48 are noncovalently associated with tyrosine kinase activity and tyrosine kinase substrates

The association of kinases and kinase substrates with Thy-1 and CD48 were characterized by immune complex kinase assays. Assays for Thy-1 antigen showed a consistent pattern of labeled proteins in rat thymocytes (Fig. 1A, track 3), mouse EL4 cells (Fig. 1B, track 3) and mouse thymocytes (Fig. 2B compares the three directly). This pattern is clearly distinct from that seen with other kinase-associated proteins (for example CD4 which shows only the band of $p56^{lck}$, Fig. 1A, track 4). Labeled bands corresponding to molecular masses of approximately 85, 60, 56, 36, and 32 kDa (sometimes a doublet) were consistently seen, and in rat thymocytes an additional band of approximately 22 kDa was often seen (Fig. 1A, track 3).

To identify these associated phosphoproteins, reprecipitation with a variety of antibodies was performed. Anti-PI3 kinase and anti-GAP antibodies failed to reprecipitate bands (Fig. 1B, tracks 4 and 5), as did anti-$p95^{vav}$ antibodies (data not shown). However, reprecipitation of Thy-1-associated proteins from EL4 cells with anti-phosphotyrosine antibodies (Fig. 1B, track 6) reproduced the original pattern of bands with exception of the 36-kDa protein, which is presumably an indication of serine/threonine phosphorylation in this protein, and infers coprecipitated serine/threonine kinase activity. The pattern of phosphorylated proteins was very similar between rat and mouse cells (Fig. 2B) although there were differences in the distribution of the 50–60-kDa bands. Rat cells have a strong 56-kDa band together with a slightly higher molecular mass band at about 60-kDa. Both mouse thymocytes and EL4 cells have this 60-kDa band together with another running at about 50-kDa. Although it is difficult to resolve, there seems to be a relative lack of a 56-kDa band.

In resting thymocytes and EL4 cells, CD48 was associated only with very weak kinase activity (Fig. 1A, track 2; Fig. 1B, track 1). However, in Con A-stimulated thymocyte blasts, a pattern similar to that of Thy-1 on resting cells was seen (Fig. 1C, track 1, and Fig. 3B and see Sect. 3.3). CD48 was also associated with kinase activity in spleen cells (Fig. 3C).

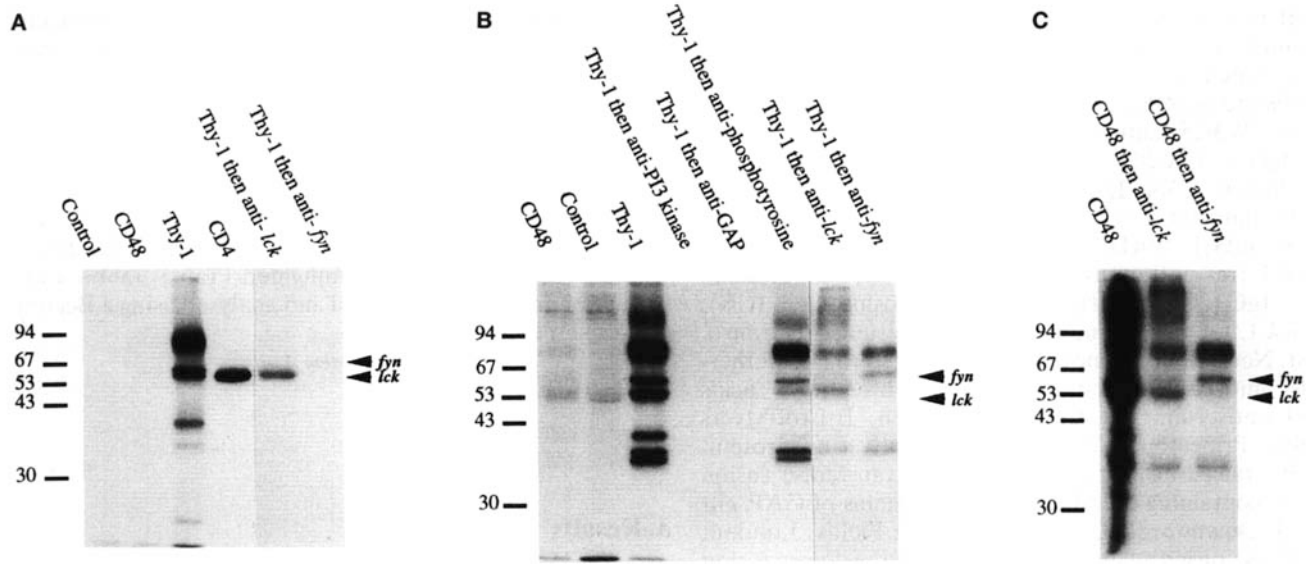

Figure 1. Association of kinase activity with Thy-1 and CD48. Panel A. Results of immunoprecipitations and *in vitro* kinase assays in rat thymocytes; 20 µl (equivalent of 1.65×10^8 cells) was loaded in each track. Track 1, OX-21 (negative control); 2, OX-45 (anti-CD48); 3, OX-7 (anti-Thy-1); 4, W3/25 (anti-CD4); 5, reprecipitation of Thy-1 assay with antiserum against $p56^{lck}$; 6, reprecipitation of Thy-1 assay with antiserum against $p60^{fyn}$. Autoradiograph was exposed for 18 h. Panel B. Results of kinase assays in mouse EL4 cells; 20 µl (equivalent of 1.3×10^7 cells) was loaded in each track. Track 1, C10 (anti-CD48); 2, OX-20 (negative control); 3, 30H12 (anti-Thy-1); 4, reprecipitation from Thy-1 assay with antiserum against the p85 subunit of PI3 kinase; 5, reprecipitation from Thy-1 assay with polyclonal IgG against GAP; 6, reprecipitation from Thy-1 assay with PY20 (anti-phosphotyrosine); 7, reprecipitation of Thy-1 assay with antiserum against $p56^{lck}$; reprecipitation of Thy-1 assay with antiserum against $p60^{fyn}$. Autoradiograph was exposed for 16 h. Panel C. Results of kinase assays in rat thymocyte Con A blasts. 1, OX-45 (anti-CD48); 2 µl (equivalent of 3×10^6 cells) was loaded; 2, reprecipitation from CD48 assay with antiserum against $p56^{lck}$, 20 µl was loaded (equivalent of 3×10^7 cells); 3, reprecipitation from CD48 assay with antiserum against $p60^{fyn}$, 20 µl was loaded, autoradiograph was exposed for 16 h.

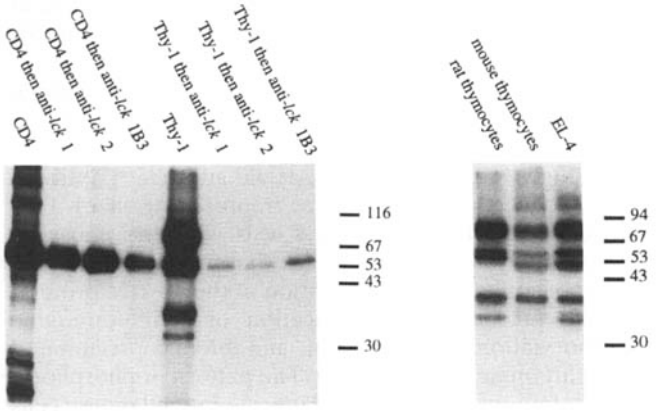

Figure 2. Reprecipitation of $p56^{lck}$ from CD4 and Thy-1 *in vitro* kinase assays in rat thymocytes. Panel A. Results from rat thymocytes. The loadings were adjusted to give approximately equal protein concentrations, as judged by Coomassie staining of gels. Track 1, result of immunoprecipitation and kinase assay with W3/25 (anti-CD4); 2 µl was loaded; 2, reprecipitation from CD4 assay using anti-$p56^{lck}$ antiserum 1, 4 µl was loaded; 3, reprecipitation using anti-$p56^{lck}$ antiserum 2, 2 µl was loaded; 4, reprecipitation with anti-$p56^{lck}$ monoclonal antibody 1B3, 10 µl was loaded; 5, immunoprecipitation and kinase assay using OX-7 (anti-Thy-1), 10 µl was loaded: 6, 7, and 8, reprecipitations from Thy-1 assays using anti-$p56^{lck}$ antibodies as in 2, 3, and 4, loading 20, 10, and 40 µl respectively. The autoradiograph exposed for 18 h. Panel B. Thy-1 kinase assays from: 1, rat thymocytes; 2, mouse thymocytes; 3, mouse EL4 cells. Each track contained an equivalent of 2×10^7 cells, the autoradiograph was exposed for 16 h.

3.2 Thy-1 and CD48 are associated with both $p56^{lck}$ and $p60^{fyn}$ in rat and mouse cells

Reprecipitation from Thy-1 and CD48 kinase assays with anti-$p56^{lck}$ and $p60^{fyn}$ antibodies produced bands of the expected sizes (Fig. 1A, tracks 5 and 6; Fig. 1B, tracks 7 and 8, and Fig. 1C, tracks 2 and 3) although in some cases other contaminating bands, particularly the prominent 85-kDa band were seen. There are conflicting reports as to the association of *src* family kinases with Thy-1 [17, 18], specifically as to whether $p56^{lck}$ is a significant component of these complexes. The 56-kDa band was confirmed to be $p56^{lck}$, by performing further reprecipitations with three different anti-p56 antibodies (Fig. 2A, tracks 6–8).

3.3 Thy-1 and CD48-associated kinase activity is modulated during Con A stimulation

When kinase assays were performed on rat thymocytes following varying periods of Con A stimulation, Thy-1 and CD48 activity showed a reciprocal relationship, with activity associated with Thy-1 being sharply down-regulated after 6 h and being very weak at 2 days. Although surface expression of Thy-1 was down-regulated during stimulation (Fig. 4A, C, E, G), this was not obvious until day 3 of stimulation, clearly lagging behind the reduction in kinase activity. CD48-associated activity, in contrast, was increased after 2 days (Fig. 3A, and B) although this might be

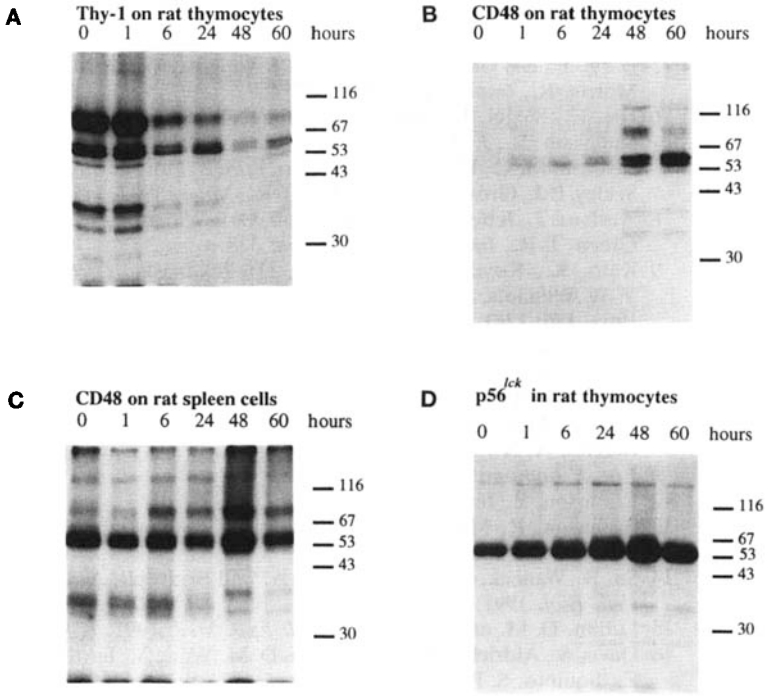

Figure 3. Association of kinase activity with Thy-1 and CD48 in rat cells during Con A stimulation. In each panel, track 1 from unstimulated cells; 2, after 1 h of stimulation; 3, after 6 h; 4, after 24 h; 5, after 48 h; 6, after 60 h. Panel A, thymocytes assayed with OX-7 (anti-Thy-1); B, thymocytes assayed with OX-45 (anti-CD48); C, spleen cells with OX-45; D, thymocytes assayed with anti-p56^{lck} antiserum 1. An equivalent of 3×10^7 cells was loaded in each case, and the autoradiograph was exposed for 16 h.

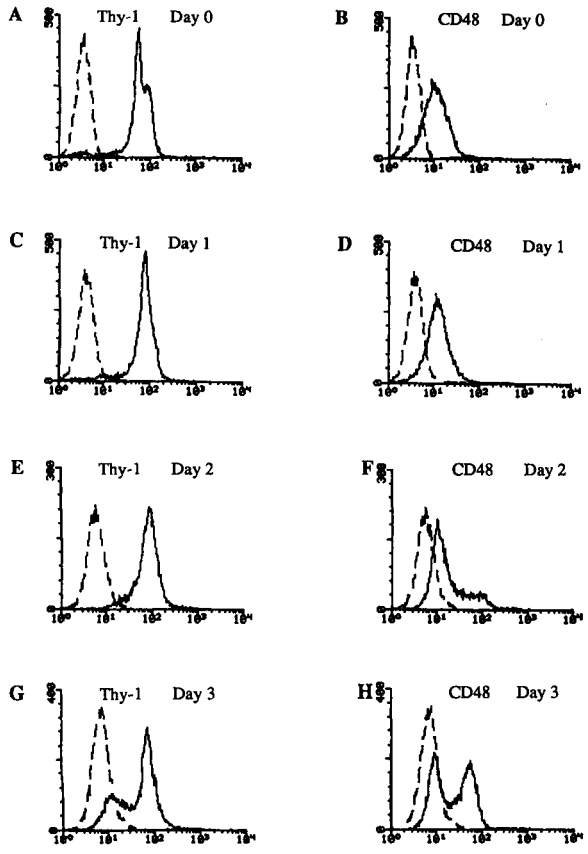

Figure 4. Surface expression of Thy-1 and CD48 by rat thymocytes during stimulation with Con A, measured by flow cytometry. A, C, E, G, expression of Thy-1 on unstimulated cells and after 1, 2, and 3 days, respectively. B, D, E, F, corresponding measurements of CD48 expression. In ech case, the dashed line represents labeling using the isotype-matched negative control antibody, OX-21.

explained by the increased surface expression of CD48 after 2 days of activation (Fig. 4F) combined with the increased levels of total p56^{lck} activity as measured by direct precipitation (Fig. 3D). A similar time course with CD48 on spleen cells showed a small increase in activity from a much higher baseline (Fig. 4C). Thy-1 is not expressed on peripheral lymphocytes in the rat [26]. Comparing the pattern of bands from Thy-1 and CD48, the Thy-1-associated 85-kDa band is consistently stronger than the 50–60-kDa complex on resting thymocytes, but this reversed as the total activity drops during stimulation. In the case of CD48, the 50–60-kDa complex is stronger than the 85-kDa complex.

4 Discussion

Recent reports have implicated either p56^{lck} or p60^{fyn} as being associated with GPI-anchored molecules on lymphocytes [17, 18]. Our data show that both are complexed to Thy-1 and CD48 on rat and mouse cells. In mouse EL4 cells and thymocytes, the p56^{lck} band was weak, although still detectable by reprecipitation, while p60^{fyn} is clearly present. However, these assays depend on the availability of free sites for phosphorylation as well as the amount and activity of kinase present and hence the strength of bands present cannot be simply correlated with amount of kinase present. It is not known whether the unidentified phosphoproteins are also kinases and these include one phosphoprotein in the same molecular weight range as p56^{lck} and p60^{fyn} in both our results and those of Thomas and Samelson [18], which appears not to reprecipitate with either anti-p56^{lck} or anti-p60^{fyn}.

Activation of thymocytes with Con A would be expected to cause marked changes in the level of kinase and phospha-

tase activity and a significant reduction was observed in Thy-1-associated phosphoproteins. Of particular interest is that this reduction is not seen with CD48 on the same cells, raising the possibility of regulated coupling of kinases with particular GPI-anchored proteins, in addition to the more general co-localization of kinases and GPI-anchored proteins in sphingolipid-rich, detergent -resistant areas of cell membrane [27, 28]. The mechanism of this regulated and specific coupling of kinases to GPI-anchored molecules is unknown, but is likely to involve other components of the multimolecular complexes demonstrated here.

Most models of signalling through GPI-anchored proteins propose their inclusion in multimolecular complexes [12] and there is now a considerable volume of evidence for associated proteins, including cytoskeletal elements [29], a coprecipitating 50-kDa or 55-kDa protein with both Thy-1 and CD48 [30–32]. The previously described Thy-1-associated 55-kDa protein associates in an approximate molar ratio of 1:15, but is unlikely to represent any *src*-like kinase since it is labeled by surface iodination of intact cells [31]. It seems very likely that these 50-kDa and 55-kDa proteins represent the same molecule partially characterized and termed Thy-3 [33]. The description of a 32-kDa membrane protein capable of binding the myristylated amino terminus of $p60^{v\text{-}src}$ suggests a possible mechanism by which GPI-anchored proteins might associate with such myristylated intracellular kinases [34]. Whether such a protein would recognize some part of the GPI structure itself, or a membrane-proximal part of the protein directly, or whether it would require another component of the complex as an intermediate remains to be seen. Another 32-kDa phosphoprotein, reported to have GTPase activity, has been coprecipitated with CD4- and CD8-$p56^{lck}$ [35]. The failure to reprecipitate the tyrosine phosphorylated 85-kDa subunit of PI3 kinase or $p95^{vav}$ with a variety of antibodies appears to rule out two obvious candidates for the abundant 85-kDa band. Thomas and Samelson [18] report a major 70–75-kDa phosphoprotein associated with murine Thy-1, which they identify as murine C-type virus envelope glycoprotein gp70. The 85-kDa band we see is present in thymocytes from both specific pathogen-free rats and mice as well as several cell lines and investigations are proceeding to identify it.

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