Protein-tyrosine kinases are known regulators of cell division that have been implicated in the onset of a variety of malignancies. They act through cellular signaling proteins that bind to specific autophosphorylation sites. To find out whether these autophosphorylation sites can be used to identify downstream signaling proteins, synthetic peptides based on an autophosphorylation site in the colony-stimulating factor-1 (CSF-1) receptor were linked to agarose beads and incubated with lysates from macrophages. Bound proteins were analyzed by MS, leading to the identification of both known and novel CSF-1 receptor-interacting proteins. The approach presented here can be applied to phosphorylation sites in a wide variety of proteins. It will lead to the identification of novel protein-protein interactions and provide new insights into the mechanics of signal transduction. Novel protein-protein interactions may provide useful targets for the development of drugs that interfere with the activation of signaling cascades used by protein-tyrosine kinases to turn on cell division.

Molecular & Cellular Proteomics 3:887–895, 2004.

The human genome encodes at least 90 different protein-tyrosine kinases (1, 2). These are involved in regulation of cell division, differentiation, adhesion, migration, metabolism, and gene transcription (1). Expression of constitutively active protein-tyrosine kinases results in uncontrolled cell division, and mutant protein-tyrosine kinases have been implicated as dominant oncogenes in a large variety of human cancers (3, 4). Consequently, protein-tyrosine kinases have been selected as targets for the development of anti-cancer drugs (5–7).

Protein-tyrosine kinases are activated directly or indirectly in response to extracellular signals and translate that information into the activation of intracellular signal transduction pathways (8, 9). Most protein-tyrosine kinases do so by using autophosphorylation sites that act as binding sites for downstream signaling proteins (8, 10). Signaling proteins, in turn, contain protein-interaction modules, such as Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains, that bind to phosphorylated tyrosine residues (8–10). The specificity of these interactions depends on the protein-interaction domain as well as on the amino acid sequence surrounding the phosphorylation site. Signaling proteins that are activated downstream of protein-tyrosine kinases may provide useful targets for the development of anti-cancer drugs. In theory, these targets can be identified using the binding sites that are present on their upstream activators.

Most signaling proteins identified thus far use SH2 or PTB domains to bind to upstream protein-tyrosine kinases. The specificity of many of these domains has been characterized in detail and generally involves only residues present immediately upstream or downstream of the phosphorylation site (11–14). To identify potential binding proteins for newly identified tyrosine phosphorylation sites, a common bioinformatics approach has been established. This approach is based on comparison of the amino acid sequence surrounding a tyrosine phosphorylation site with the specificity of known SH2 and PTB domains. In vitro binding and co-immunoprecipitation experiments are subsequently carried out to test the prediction. The disadvantage of this approach is that it considers only known signaling proteins containing well-characterized protein-protein interaction domains. Here we have demonstrated the use of peptides based on tyrosine phosphorylation sites as affinity regents to identify protein-tyrosine kinase-interacting proteins and substrates. This approach has yielded both known signaling proteins and proteins with no previously known signaling function.

EXPERIMENTAL PROCEDURES

Cell Culture—Murine P388D1 macrophages were grown in Iscove’s-modified Dulbecco’s medium supplemented with 10% fetal bovine serum at 37 °C in 10% CO2 until confluent and then starved for 12 h in serum-free medium. Control or colony-stimulating factor-1 (CSF-1)-stimulated cells (200 ng/ml) were lysed in 1 ml of phospholipase C (PLC) lysis buffer per 10-cm dish (15).

1 The abbreviations used are: SH2, Src homology 2; CSF-1, colony-stimulating factor 1; EGF, epidermal growth factor; PTB, phosphotyrosine binding; P.Tyr, phosphotyrosine; SH3, Src homology 3; TKB, tyrosine kinase binding; PLC, phospholipase C.
Identification of Phosphorylation Site-binding Proteins

Antisera, Immunoprecipitation, and Immunoblotting—Polyclonal antiserum against Cbl, the CSF-1 receptor, NonO, and PSF were generated as previously described (15, 16). A polyclonal anti-Shp2 serum was provided by G.-S. Feng (Burnham Institute, La Jolla, CA), a polyclonal anti-Grb2 serum was provided by T. Hunter (Salk Institute, La Jolla, CA), polyclonal sera against Sos and SHIP were purchased from Upstate Biotechnology Inc. (Waltham, MA), a polyclonal serum against Cbl, the CSF-1 receptor, NonO, and PSF were generated as previously described (15, 16), a polyclonal anti-Shp2 serum was provided by G.-S. Feng (Burnham Institute, La Jolla, CA), and a monoclonal antibody against clathrin heavy chain was purchased from BD Biosciences Pharmingen (San Diego, CA). Immunoprecipitation and immunoblotting were carried out exactly as described previously (15).

GST Fusion Proteins—GST-Cbl tyrosine kinase binding (TKB) was generated as previously described (15), GST-Grb2 SH2 was provided by T. Pawson (Samuel Lunenfeld Research Institute, Toronto, Canada), and GST-Shp2 SH2(N+1) was provided by G.-S. Feng (Burnham Institute). GST fusion proteins were produced and purified exactly as previously described (15).

Affinity Purification of CSF-1 Receptor-binding Proteins—Phosphorylated and unphosphorylated peptides based on sequences surrounding Tyr-544 (TYKQKPKYQVRWKI), Tyr-706 (IHLEKKYVRRD- SG), and Tyr-973 (EPRDQSSTLP SQC---HQQI LQQQQMVNEE) were synthesized by Synpep (Dublin, CA). The Tyr-544 and Tyr-706 peptides were synthesized with a cysteine residue followed by an aminohexanoic acid attached to the amino terminus. The cysteine was used for coupling to SulfoLink (Pierce, Rockford, IL); the aminohexanoic acid was added to provide spacing between the peptide and the support material. The Tyr-973 peptides were synthesized with anaminohexanoic acid attached to the amino terminus and coupled to cyano gen bromide-Sepharose (Sigma, St. Louis, MO). PLC lysates were incubated batch-wise with immobilized peptides at 4 °C on a rocker. The beads were washed four times with PLC lysis buffer and bound proteins were analyzed by silverstaining or MS.

MS and Database Searching—Protein bands were excised from a polyacrylamide gel stained with GelCode Blue (Pierce). After destaining and neutralization, protein bands were reduced and alkylated using DTT (Sigma) and iodoacetic acid (Aldrich, Milwaukee, WI). The bands were then washed and digested overnight at 37 °C with sequencing grade, modified trypsin (Promega). Peptides were extracted in 5% ACN + 0.1% acetic acid and desalted on C18 ZipTips (Millipore, Bedford, MA) and eluted into 70% ACN + 0.1% acetic acid. Approximately 5 μl of the 10-μl peptide extract was loaded into a nanospray capillary (Proxeon Biosystems A/S, Odense, Denmark) (17–20). Mass spectra of the peptide extracts from 400 to 1,500 m/z were collected on an Applied Biosystems QSTAR hybrid QTOF mass spectrometer (Foster City, CA) equipped with a Proxeon Biosystems A/S nanospray source. Prominent doubly and triply charged tryptic peptide ions were selected for fragmentation and MS/MS spectra were recorded. Peptide masses, fragment ion masses and short sequence tags were used to search both the Swiss-Prot and nonredundant databases for murine proteins that matched the tryptic peptide mass and sequence data. Spectral analysis and database searching were carried out using Applied Biosystems Analyst and BioAnalyzer suite of programs on the QSTAR.

RESULTS

The Carboxyl Terminus of the CSF-1 Receptor BindsSpecifically to a Variety of Proteins Present in Macrophage Cell Lysates—The CSF-1 receptor is a protein-tyrosine kinase that is expressed on the surface of macrophages and macrophage precursors (21–24). Binding of CSF-1 to the extracellular ligand-binding domain results in activation of the kinase domain and autophosphorylation on a number of tyrosine residues, including Tyr-973 (15, 25). In contrast to most of the region carboxyl terminal to the kinase domain, residues immediately upstream and downstream of Tyr-973 have been highly conserved during evolution (Fig. 1). This suggests that the carboxyl terminus performs an evolutionarily conserved function that is likely to involve multiple protein-protein interactions. To test this hypothesis, phosphorylated and unphosphorylated peptides based on the sequence around Tyr-973 were used as affinity reagents to purify CSF-1 receptor-interacting proteins. Peptides were immobilized on agarose beads and incubated with lysates of control and CSF-1-stimulated macrophages. Bound proteins were analyzed by silverstaining (Fig. 2A). The results show that there are various proteins that bind specifically to the unphosphorylated peptide, while oth-

FIG. 1. The sequence around Tyr-973 in the human CSF-1 receptor has been conserved during evolution. A, schematic representation of the CSF-1 receptor. The receptor contains an extracellular ligand binding domain, a single transmembrane helix, a kinase domain (KD) that is divided into two parts by a kinase insert region, and a carboxyl-terminal region. Relevant autophosphorylation sites (Y) are indicated. B, alignment of the carboxyl termini of CSF-1 receptors from human (Hs), mouse (Mm), zebrafish (Dd), rainbow trout (Om), and pufferfish (Tr). Residues that have been conserved in all five species are boxed. The end of the kinase domain is indicated.
ers bind specifically to the phosphorylated peptide (Fig. 2A). Many more proteins bound to the Tyr-973 peptides. These results show that peptides can be used to purify protein-tyrosine kinase-binding proteins. The array of proteins that bind to a particular peptide is determined by its sequence, and both phosphorylation-dependent and independent interactions can be observed.

Isolation of P.Tyr-containing Proteins Using the P.Tyr-973 Peptide—To investigate their phosphorylation status, peptide-bound proteins were analyzed by anti-P.Tyr immunoblotting. Whole-cell lysates were analyzed in parallel. The results show that there are several tyrosine-phosphorylated proteins that bind specifically to the phosphorylated Tyr-973 peptide (Fig. 2B). Some of these proteins were phosphorylated in control as well as stimulated cells, while others were tyrosine-phosphorylated only in CSF-1-stimulated cells (Fig. 2B). This shows that proteins that are tyrosine-phosphorylated in response to CSF-1 can be isolated using the phosphorylated Tyr-973 peptide.

Identification of Tyr-973-binding Proteins as Mediators of CSF-1 Receptor Signal Transduction—Lysates from 2–5 × 10⁶ P388D1 macrophages were incubated with agarose beads containing either the phosphorylated or unphosphorylated Tyr-973 peptide. Bound proteins were resolved by SDS-PAGE and visualized by GelCode Blue staining (Fig. 3). Prominent protein bands were isolated from the gel, digested with trypsin, and analyzed by MS. Peptide masses, fragment ion masses, and short sequence tags were used to search databases. Proteins were identified based on tryptic peptide mass and sequence data. Both proteins and peptides that were identified are shown (Fig. 3). We identified four proteins that bind specifically to the unphosphorylated Tyr-973 peptide: clathrin heavy chain, a protein involved in pinching off membrane vesicles; PSF, a transcription and splicing factor; HSC73, a heat-shock protein-like protein; and NonO, a transcription factor (26–29). In addition, we identified five proteins that bound to the phosphorylated Tyr-973 peptide: Sos1, an activator of Ras; two isoforms of SHIP, a phosphatidylinositol phosphatase; Cbl, a ubiquitin ligase; Shp1, a protein-tyrosine phosphatase; and Grb2, an adaptor protein (30–35).

Sos, SHIP, Cbl, Shp1, and Grb2 have been implicated previously in CSF-1 receptor signaling (Table I). To confirm that they bind specifically and in a phosphorylation-dependent manner to Tyr-973, lysates of P388D1 macrophages were incubated with agarose beads containing either the phosphorylated or unphosphorylated Tyr-973 peptide. Bound proteins were analyzed by SDS-PAGE and visualized by GelCode Blue staining (Fig. 3). Prominent protein bands were isolated from the gel, digested with trypsin, and analyzed by MS. Peptide masses, fragment ion masses, and short sequence tags were used to search databases. Proteins were identified based on tryptic peptide mass and sequence data. Both proteins and peptides that were identified are shown (Fig. 3). We identified four proteins that bind specifically to the unphosphorylated Tyr-973 peptide: clathrin heavy chain, a protein involved in pinching off membrane vesicles; PSF, a transcription and splicing factor; HSC73, a heat-shock protein-like protein; and NonO, a transcription factor (26–29). In addition, we identified five proteins that bound to the phosphorylated Tyr-973 peptide: Sos1, an activator of Ras; two isoforms of SHIP, a phosphatidylinositol phosphatase; Cbl, a ubiquitin ligase; Shp1, a protein-tyrosine phosphatase; and Grb2, an adaptor protein (30–35).

Sos, SHIP, Cbl, Shp1, and Grb2 have been implicated previously in CSF-1 receptor signaling (Table I). To confirm that they bind specifically and in a phosphorylation-dependent manner to Tyr-973, lysates of P388D1 macrophages were incubated with Tyr-973 or P.Tyr-973 peptide agarose beads and bound proteins were analyzed by immunoblotting. The results show that Sos, SHIP, Cbl, Shp1, and Grb2 all bind specifically to the phosphorylated but not to the unphosphorylated Tyr-973 peptide (Fig. 4A). Shp2, a protein-tyrosine phosphatase that is closely related to Shp1, has also been implicated in CSF-1 receptor signal transduction (Table I). To test whether Shp2 can also bind to the Tyr-973 phosphorylation site, P388D1 lysates were incubated with immobilized Tyr-973 peptides and bound proteins were analyzed by anti-Shp2 immunoblotting (Fig. 4A). The results show that Shp2
also binds specifically to the phosphorylated Tyr-973 peptide. Thus Sos, SHIP, Cbl, Shp1, Shp2, and Grb2 all bind specifically to the phosphorylated Tyr-973 peptide.

To find out whether binding was direct, purified GST fusion proteins containing the Cbl TKB domain, the tandem Shp2 SH2 domains, or the Grb2 SH2 domain were tested for their ability to bind to the Tyr-973 and P.Tyr-973 peptides (Fig. 4B). All three fusion proteins bound specifically to the phosphorylated Tyr-973 peptide (Fig. 4B). No binding to the unphosphorylated Tyr-973 peptide or the control peptides was observed. These observations suggest that Cbl, Shp2, and Grb2 bind directly and specifically to Tyr-973 in a phosphorylation-dependent manner.

Neither NonO nor PSF has been implicated previously in CSF-1 receptor signaling. To confirm that nonO binds specifically to Tyr-973, lysates of unstimulated P388D1 macrophages were incubated with immobilized Tyr-973 peptides and bound proteins were analyzed by anti-NonO immunoblotting. The results show that PSF and NonO associate with unphosphorylated CSF-1 receptors isolated from P388D1 macrophages.

To confirm that clathrin associates with Tyr-973, lysates of unstimulated P388D1 macrophages were incubated with immobilized Tyr-973 peptides. Peptides based on the sequence around Tyr-706 were included as controls. Bound proteins were analyzed by anti-clathrin immunoblotting (Fig. 6). The results show that the clathrin heavy chain associates specifically with the unphosphorylated Tyr-973 peptide.

**DISCUSSION**

Substrates and downstream signaling proteins are recruited to activated protein-tyrosine kinases through binding to specific autophosphorylation sites (8–10, 36). To identify binding partners for tyrosine phosphorylation sites, several approaches have been used in the past. Margolis and co-
workers pioneered the use of the carboxyl terminus of the epidermal growth factor (EGF) receptor to screen cDNA expression libraries (35, 37). In this experiment, the recombinant EGF receptor cytoplasmic domain was labeled by autophosphorylation in the presence of \( \gamma^{32}P \)ATP. Subsequently, the carboxyl terminus is separated from the remainder of the protein and used to probe a cDNA expression library. This approach has been used successfully to identify a large number of growth factor receptor-binding proteins (35, 37, 38). However, it is cumbersome and has not been used to identify binding partners for specific phosphorylation sites. In addition, it is unclear whether this approach can be used to identify binding partners for other protein-tyrosine kinases.

A second approach is based on the yeast two-hybrid screen. In this approach, the kinase of interest is fused to the DNA-binding domain of a transcription factor (39). Expression of proteins that bind to the kinase of interest results in reconstitution of a transcription factor that drives the expression of a selectable marker. Two-hybrid screens have been used successfully to identify mediators of protein-tyrosine kinase signaling (32, 39). However, investigators have encountered problems with the expression of specific protein-tyrosine kinases and with identification of false positives. The two-hybrid screen may not be the ideal tool to identify binding partners for specific phosphorylation sites.

As a complement to these approaches, we have used phosphopeptides as affinity reagents to purify binding partners for specific tyrosine phosphorylation sites. Because this method is based on synthetic peptides that are stoichiometrically phosphorylated at a specific site, this method can be applied to any phosphorylation site of interest. Peptides can
be immobilized at high density on agarose beads using standard chemistry. As a consequence, this method can be used to obtain sufficient amounts of relatively rare proteins for identification by MS. We have used peptides based on the sequence surrounding Tyr-973 in the carboxyl terminus of the CSF-1 receptor. This led to the identification of four proteins that bind to the unphosphorylated peptide and six proteins that bind to the phosphorylated peptide. In a similar approach, peptides based on the sequence surrounding Tyr-1068 in the EGF receptor were used, leading to the identification of Grb2 (40). These experiments demonstrate that phosphopeptide-based protein purification combined with MS can be used as a powerful tool to identify binding partners for receptor protein-tyrosine kinases.

Four of the six proteins that bind to the phosphorylated Tyr-973 peptide, including Cbl, SHIP, Shp1, and Shp2, are likely to act as negative regulators of CSF-1-dependent cell division. The first one, c-Cbl, is a ubiquitin ligase composed of an amino-terminal TKD domain, a ring-finger, a proline-rich region, and a carboxyl terminus containing several tyrosine phosphorylation sites (41). Cbl is thought to cause degradation of activated CSF-1 receptors (42–44). It was found recently that c-Cbl binds directly to the carboxyl terminus of the activated CSF-1 receptor. Identification of c-Cbl shows that the approach used here yields physiologically relevant receptor-binding proteins.

We found that in addition to Cbl, the lipid phosphatase SHIP can also bind to the phosphorylated CSF-1 receptor carboxyl terminus. SHIP is a 150-kDa protein that is composed of an amino-terminal SH2 domain, a central lipid phosphatase domain, and a carboxyl terminus containing several tyrosine phosphorylation sites (45, 46). SHIP is tyrosine-phosphorylated in response to CSF-1, and there is good evidence that suggests that SHIP is a negative regulator of CSF-1 receptor signaling (32, 46, 47). Our results show that SHIP interacts with the CSF-1 receptor either directly using its SH2 domain or indirectly through Grb2.

Finally, we found that both the SH2 domain-containing protein phosphatases, Shp1 and Shp2, can bind to the Tyr-973 phosphorylation site. Shp1 is known to be tyrosine-phosphorylated following stimulation of macrophages with CSF-1 (48, 49). Shp1-deficient mice have increased numbers of macrophages, and Shp1-deficient macrophages show an increase in the rate of proliferation following stimulation with CSF-1 (50–52). These observations suggest that Shp1 is a negative regulator of CSF-1 receptor signaling. Shp2 is a protein-tyrosine phosphatase that is closely related to Shp1 (34). Shp2 is known to act in concert with Gab2 to inhibit cell division and promote differentiation in response to CSF-1 (53, 54). Thus, there are four negative regulators of CSF-1-dependent proliferation that can interact specifically with the carboxyl terminus of the CSF-1 receptor. This is of interest because the oncogenic potential of CSF-1 receptor mutants is known to be enhanced by a carboxyl terminal deletion (55–57). Our data suggests that the carboxyl terminal deletion results in an increase in oncogenic potential by preventing the activated CSF-1 receptor mutants from interacting with Cbl, SHIP, Shp1, or Shp2.

In contrast to Cbl, SHIP, Shp1, and Shp2, the Grb2-Sos complex is thought to be involved in sustaining DNA synthesis and cell division in response to CSF-1 (46, 58). Grb2 uses its SH2 domain to interact with activated receptors and its SH3 domains to associate with either Sos or Gab1 (59, 60). Tyr-697 had been identified previously as a binding site for Grb2 on the CSF-1 receptor (58). Interestingly,
mutation of Tyr-697 does not completely block the interaction between the receptor and Grb2, suggesting that there may be a second binding site (46, 61). Our observations suggest that Tyr-973 may represent a second binding site for the Grb2 on the CSF-1 receptor. Identification of Sos as a P.Tyr-973-binding protein illustrates that the method used here can also result in the isolation of proteins that bind to a phosphorylation site indirectly.

Clathrin heavy chain, which was identified as a protein that binds to the unphosphorylated Tyr-973 peptide, is a 180-kDa protein that is involved in the formation of membrane-enclosed vesicles (26). The clathrin heavy chain acts together with the light chain and specific adaptor complexes to initiate the formation of coated pits and, subsequently, coated vesicles. This process is important for endocytosis and transport of proteins and lipids between compartments in the cell. Clathrin associates with the membrane through adaptor proteins or complexes that bind to transmembrane proteins present in the coated pits or vesicles (62). Adaptor proteins or complexes usually recognize NPXY or YXXΦ motifs (62, 63). Tyr-973 is present within a sequence that loosely conforms to the YXXΦ motif (Fig. 1A). Similar to what has been found in many other clathrin-binding proteins, this motif is present at the carboxyl terminus of the CSF-1 receptor (63). These observations suggest that clathrin associates with the CSF-1 receptor indirectly through an adaptor complex. Finally, clathrin binds exclusively to the unphosphorylated Tyr-973 peptide. This suggests that this interaction is not involved in the internalization of activated receptors but instead functions in transport of inactive receptors within the cell. This is consistent with observations showing large numbers of CSF-1 receptors associated with cytoplasmic vesicles in unstimulated macrophages (64).

NonO and PSF are two related proteins that co-purify in a complex and that have been implicated in RNA splicing and the activation of gene transcription (65, 66). Identification of NonO and PSF is of interest because there is increasing evidence that receptor protein-tyrosine kinases can be subject to intramembrane cleavage followed by release of their cytoplasmic domain into the cytosol and migration into the nucleus (64, 67, 68). Thus, we have identified a variety of proteins that appear to interact specifically with the carboxyl terminus of the CSF-1 receptor. The notion that the carboxyl terminus of the CSF-1 receptor acts as a docking site for several signaling proteins with diverse functions is consistent with the fact that it is highly conserved during evolution (Fig. 1A).

We have shown that peptides based on tyrosine phosphorylation sites are powerful affinity reagents that can be used for the purification and identification of signaling molecules. Identification of these proteins will lead to new insights in signal transduction. In addition, they may provide targets for the development of drugs that interfere with protein-tyrosine kinase signaling. Finally, we think that this approach may be used to enhance the sensitivity of diagnostic tests and believe that it can also be used for the identification of binding partners for serine- and threonine-based phosphorylation sites.

* This work was supported in part by Grant CA78629 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ K. W. and J. C. contributed equally to this study.

| To whom correspondence should be addressed: Department of Chemistry and Biochemistry, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92039-0601. Tel.: 858-822-2024; Fax: 858-822-0079; E-mail: geer@ucsd.edu. |

REFERENCES

1. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. Science 298, 1912–1934
2. Robinson, D. R., Wu, Y. M., and Lin, S. F. (2000) The protein tyrosine kinase family of the human genome. Oncogene 19, 5548–5557
3. Blume-Jensen, P., and Hunter, T. (2001) Oncogenic kinase signalling. Nature 411, 355–365
4. Kolliba, K. S., and Druker, B. J. (1997) Protein tyrosine kinases and cancer. Biochem. Biophys. Acta 1333, F217–248
5. Fabbro, D., Parkinson, D., and Matter, A. (2002) Protein tyrosine kinase inhibitors: new treatment modalities? Curr. Opin. Pharmacol. 2, 374–381
6. Al-Obeidi, F. A., and Lam, K. S. (2000) Development of inhibitors for protein tyrosine kinases. Oncogene 19, 5690–5701
7. Cohen, P. (2002) Protein kinases—The major drug targets of the twenty-first century? Nat. Rev. Drug Discov. 1, 309–315
8. Pawson, T. (1995) Protein modules and signalling networks. Nature 373, 573–580
9. Pawson, T., Gish, G. D., and Nash, P. (2001) SH2 domains, interaction modules and cellular wiring. Trends Cell Biol. 11, 504–511
10. van der Geer, P., Hunter, T., and Lindberg, R. A. (1994) Receptor protein-tyrosine kinases and their signal transduction pathways. Annu. Rev. Cell Biol. 10, 251–337
11. Margolis, B., Borg, J. P., Straight, S., and Meyer, D. (1999) The function of PTB domain proteins. Kidney Int. 56, 1230–1237
12. Songyang, Z., Margolis, B., Chaudhuri, M., Shoelson, S. E., and Cantley, L. C. (1995) The phosphotyrosine-protein interaction domain of SHC recognizes tyrosine-phosphorylated NPXY motifs. J. Biol. Chem. 270, 14863–14866
13. Songyang, Z., Shoelson, S. E., Clague, J., Olivier, P., Pawson, T., Bustin, J., Baracid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnoffsky, S., Feldman, R. A., and Cantley, L. C. (1994) Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav (5557–5560)
14. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnoffsky, S., Schleichere, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) SH2 domains recognize specific phosphopeptide sequences. Cell 72, 787–778
15. Wilhelmsson, K., Burkharter, S., and van der Geer, P. (2002) C-Brt binds the CSF-1 receptor at tyrosine 973, a novel phosphorylation site in the receptor’s carboxy-terminus. Oncogene 21, 1079–1089
16. Mathur, M., Tucker, P. W., and Samuels, H. H. (2001) PSF is a novel co-repressor that mediates its effect through Sin3A and the DNA binding domain of nuclear hormone receptors. Mol. Cell. Biol. 21, 2398–2311
17. Speicher, K., Kolbas, O., Harper, S., and Speicher, D. (2002) Cytosolic localization and functional analysis of peptide recoveries from in-gel digestions for protein identification in proteome studies. J. Biolum. Chem. 11, 74–86
18. Rosenfeld, J., Capdevielle, J., Guillotom, J. C., and Ferrara, P. (1992) In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal. Biochem. 203, 173–179
19. Hellman, U., Wernstedt, C., Gonez, J., and Heldin, C. H. (1995) Improvement of an “in-gel” digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. Anal. Biochem. 224, 451–455
Identification of Phosphorylation Site-Binding Proteins

20. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850–858

21. Cossens, L., van Zijl, C., Smith, D., Chen, E., Mitchell, R. L., Issace, C. M., Verma, I. M., and Ullrich, A. (1986) Structural alteration of viral homologue of receptor proto-oncogene fms at carboxyl terminus. Nature 320, 277–280

22. Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T., and Stanley, E. R. (1985) The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41, 253–266

23. Rettenmier, C. W., Sacca, R., Furman, W. L., Roussel, M. F., Holt, J. T., Nienhuis, A. W., Stanley, E. R., and Sherr, C. J. (1986) Expression of the human c-fms proto-oncogene product (colony-stimulating factor-1 receptor) on peripheral blood mononuclear cells and chlorocarcinoma cell lines. J. Clin. Invest. 77, 1740–1746

24. Woolford, J., Rothwell, V., and Rohrschneider, L. (1985) Characterization of the human c-fms gene product and its expression in cells of the monocyte-macrophage lineage. Mol. Cell. Biol. 5, 3458–3466

25. Bourette, R. P., and Rohrschneider, L. R. (2000) Early events in M-CSF receptor signaling. Growth Factors 17, 155–166

26. Brodsky, F. M., Chen, C. Y., Knuehl, C., Towler, M. C., and Wakeham, D. E. (2001) Biological basket weaving: Formation and function of clathrin-coated vesicles. Annu. Rev. Cell Dev. Biol. 17, 517–568

27. Patton, J. G., Poro, E. B., Galceran, J., Tempst, P., and Nadal-Ginard, B. (1993) Cloning and characterization of PSF, a novel pre-mRNA splicing factor. Genes Dev. 7, 393–406

28. Sorger, P. K., and Pelham, H. R. (1987) Cloning and expression of a gene encoding hsp73, the major hsp70-like protein in unstimmed rat cells. EMBO J. 6, 993–998

29. Yang, Y. S., Hanke, J. H., Carayannopoulos, L., Craft, C. M., Capra, J. D., and Tucker, P. W. (1993) NonO, a non-POU-domain-containing, octamer-binding protein, is the mammalian homolog of Drosophila nonAdiss. Mol. Cell. Biol. 13, 5593–5603

30. Bowtell, D., Fu, P., Simon, M., and Senior, P. (1992) Identification of murine homologues of the Drosophila Son of seven gene: Potential activators of ras. Proc. Natl. Acad. Sci. U. S. A. 89, 6511–6515

31. Damen, J. E., Liu, L., Rosten, P., Humphries, R. K., Jefferson, A. B., Majerus, P. W., and Krystal, G. (1996) The 145-kDa protein that is involved to associate with Shc by multiple cytokines is an inositol tetraphosphate phosphatase and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. Proc. Natl. Acad. Sci. U. S. A. 93, 1689–1693

32. Lioubin, M. N., Algate, P. A., Tsai, S., Carlberg, K., Aebersold, R., and Rohrschneider, L. R. (1996) p150SHIP, a signal transduction molecule with SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinase signals to ras. EMBO J. 15, 5520–5526

33. Dikic, I., Szymkiewicz, I., and Soubeyran, P. (1993) Cbl signaling networks in the regulation of cell function. Cell. Mol. Life Sci. 60, 1805–1827

34. Wang, Y., Yeung, Y. G., and Stanley, E. R. (1999) CSF-1 stimulated multiquitination of the CSF-1 receptor and of Cbl follows their tyrosine phosphorylation and association with other signaling proteins. J. Cell. Biochem. 72, 119–134

35. Wang, Y., Yeung, Y. G., Langdon, W. Y., and Stanley, E. R. (1996) c-Cbl is transiently tyrosine-phosphorylated, ubiquitinated, and membrane-targeted following CSF-1 stimulation of macrophages. J. Biol. Chem. 271, 17–20

36. Lee, P. S., Wang, Y., Dominguez, M. G., Yeung, Y. G., Murphy, M. A., Bowtell, D. D., and Stanley, E. R. (1999) The Cbl protooncoprotein stimulates CSF-1 receptor multiquititation and endocytosis, and attenuates macrophage proliferation. EMBO J. 18, 3616–3628

37. Rohrschneider, L. R., Fuller, J. F., Wolf, I., Liu, Y., and Lucas, D. M. (2000) Structure, function, and biology of SHIP proteins. Genes Dev. 14, 505–520

38. Lioubin, M. N., Myles, G. M., Carlborg, K., Bowtell, D., and Rohrschneider, L. R. (1996) ShcGrb2, a 150-kidoladon tyrosine-phosphorylated protein form complexes with Fms in hematopoietic cells. Mol. Cell. Biol. 16, 5682–5691

39. Takeshita, S., Namba, N., Zhao, J. J., Jiang, Y., Genant, H. K., Silva, M. J., Brodt, M. D., Helgason, C. D., Kalesnikoff, J., Rauh, M. J., Humphries, R. K., Krystal, G., Teitelbaum, S. L., and Ross, F. P. (2002) SHIP-deficient mice are severely osteoporetic due to increased numbers of hyper-resorptive osteoclasts. Nat. Med. 8, 943–949

40. Yi, T., and Ihle, J. N. (1993) Association of hematopoietic cell phosphatase with c-kit after stimulation with c-kit ligand. Mol. Cell. Biol. 13, 3350–3358

41. Yeung, Y. G., Berg, K. L., Pixley, F. J., Angeletti, R. H., and Stanley, E. R. (1992) Protein tyrosine phosphatase-1C is rapidly phosphorylated in tyrosine in macrophages in response to colony stimulating factor-1. J. Biol. Chem. 267, 23447–23450

42. Shultz, L. D., Bailey, C. L., and Coman, D. R. (1983) Hematopoietic stem cell function in motheaten mice. Exp. Hematol. 11, 667–680

43. Shultz, L. D., Schweitzer, P. A., Rajan, T. V., Yi, T., Ihle, J. N., Matthews, R. J., Thomas, M. L., and Beier, D. R. (1993) Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. Cell 73, 1445–1454

44. Chen, H. E., Chang, S., Trub, T., and Neel, B. G. (1996) Regulation of colony-stimulating factor 1 receptor signaling by the SH2 domain-containing tyrosine phosphatase SHP-1. Mol. Cell. Biol. 16, 3685–3697

45. Carlborg, K., and Rohrschneider, L. R. (1997) Characterization of a novel tyrosine phosphorylated 100-kDa protein that binds to SHP-2 and phosphatidylinositol 3′-kinase in myeloid cells. J. Biol. Chem. 272, 15943–15950

46. Liu, Y., Jenkins, B., Shin, J. L., and Rohrschneider, L. R. (2001) Scaffolding protein Gab2 mediates differentiation signaling downstream of Fms receptor tyrosine kinase. Mol. Cell. Biol. 21, 3047–3056

47. Roussel, M. F., Dull, T. J., Rettenmier, C. W., Ralph, P., Ulrich, A., and Sherr, C. J. (1987) Transforming potential of the c-fms proto-oncogene (CSF-1 receptor). Nature 325, 549–552

48. Browning, P. J., Bunn, H. F., Cline, A., Shuman, M., and Nienhuis, A. W. (1986) “Replacement” of COOH-terminal truncation of v-fms with c-fms sequences markedly reduces transformation potential. Proc. Natl. Acad. Sci. U. S. A. 83, 7780–7785

49. Woolford, J., McAuliffe, A., and Rohrschneider, L. R. (1988) Activation of the feline c-fms proto-oncogene: Multiple alterations are required to generate a fully transformed phenotype. Cell 55, 965–977

50. van der Geer, P., and Hunter, T. (1993) Mutation of Tyr697, a GRB2-binding site for the murine fms tyrosine kinase. Oncogene 12, 5161–5172

51. Tari, A. M., and Lopez-Berestein, G. (2001) GRB2: A pivotal protein in signal transduction. Semin. Oncol. 28, 142–147

52. Lioubin, M. N., Bowtell, D., and Rohrschneider, L. R. (2000) The Gift of Gab. FEBS Lett. 515, 1–7

53. Mancini, A., Niedenthal, R., Joos, H., Koch, A., Troulis, S., Niemann, H., and Tamura, T. (1997) Identification of a second Grb2 binding site in the v-fms tyrosine kinase. Oncogene 15, 1565–1572
62. Robinson, M. S., and Bonifacino, J. S. (2001) Adaptor-related proteins. Curr. Opin. Cell Biol. 13, 444–453
63. Bonifacino, J. S., and Traub, L. M. (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu. Rev. Biochem. 72, 395–447
64. Wilhelmsen, K., and van der Geer, P. (2004) Phorbol 12-myristate 13-acetate-induced release of the colony-stimulating factor 1 receptor cytoplasmic domain into the cytosol involves two separate cleavage events. Mol. Cell. Biol. 24, 454–464
65. Zhang, W. W., Zhang, L. X., Busch, R. K., Farres, J., and Busch, H. (1993) Purification and characterization of a DNA-binding heterodimer of 52 and 100 kDa from HeLa cells. Biochem. J. 290, 267–272
66. Shav-Tal, Y., and Zipori, D. (2002) PSF and p54(nrb)/NonO—Multi-functional nuclear proteins. FEBS Lett. 531, 109–114
67. Ni, C. Y.; Murphy, M. P.; Golde, T. E., and Carpenter, G. (2001) γ-Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science 294, 2179–2181
68. Carpenter, G. (2003) Nuclear localization and possible functions of receptor tyrosine kinases. Curr. Opin. Cell Biol. 15, 143–148
69. Mancini, A., Koch, A., Wilms, R., and Tamura, T. (2002) c-Cbl associates directly with the C-terminal tail of the receptor for the macrophage colony-stimulating factor, c-Fms, and down-modulates this receptor but not the viral oncogene v-Fms. J. Biol. Chem. 277, 14635–14640
70. Kharbanda, S., Saleem, A., Yuan, Z., Emoto, Y., Prasad, K. V., and Kufe, D. (1995) Stimulation of human monocytes with macrophage colony-stimulating factor induces a Grb2-mediated association of the focal adhesion kinase pp125FAK and dynamin. Proc. Natl. Acad. Sci. U. S. A. 92, 6132–6136
71. Yeung, Y. G., Wang, Y., Einstein, D. B., Lee, P. S., and Stanley, E. R. (1998) Colony-stimulating factor-1 stimulates the formation of multimeric cytosolic complexes of signaling proteins and cytoskeletal components in macrophages. J. Biol. Chem. 273, 17128–17137
72. Saleem, A., Kharbanda, S., Yuan, Z. M., and Kufe, D. (1995) Monocyte colony-stimulating factor stimulates binding of phosphatidylinositol 3-kinase to Grb2:Sos complexes in human monocytes. J. Biol. Chem. 270, 10380–10383
73. Husson, H., Mograbi, B., Schmid-Antomarchi, H., Fischer, S., and Rossi, B. (1997) CSF-1 stimulation induces the formation of a multiprotein complex including CSF-1 receptor, c-Cbl, PI 3-kinase, Crk-II and Grb2. Oncogene 14, 2331–2338
74. Liu, S. K., Berry, D. M., and McGlade, C. J. (2001) The role of Gads in hematopoietic cell signalling. Oncogene 20, 6284–6290
75. Wolf, I., Jenkins, B. J., Liu, Y., Seiffert, M., Custodio, J. M., Young, P., and Rohrschneider, L. R. (2002) Gab3, a new DSS/Gab family member, facilitates macrophage differentiation. Mol. Cell. Biol. 22, 231–244