B Cell Antigen Receptor Signaling Induces the Formation of Complexes Containing the Crk Adapter Proteins*

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Crk proteins are Src homology (SH) 2/SH3-containing adapter proteins that can mediate the formation of signaling complexes. We show that engaging the B cell antigen receptor (BCR) on the RAMOS B cell line caused both the SH2 and SH3 domains of Crk-L to bind to several tyrosine phosphorylated proteins. We identified two of these phosphoproteins as Cas and Cbl and showed that both bound to the Crk SH2 domain after BCR engagement. BCR ligation also increased the amount of Crk proteins in the particulate fraction of the cells and induced the formation of Crk-Cas and Crk-Cbl complexes in the particulate fraction. We propose that tyrosine phosphorylation of membrane-associated Cas and Cbl creates binding sites for the Crk SH2 domain and recruits Crk complexes to cellular membranes. Thus, Crk proteins may participate in BCR signaling by using their SH2 domains to direct the interactions and subcellular localization of proteins that bind to their SH3 domains. In RAMOS cells, we found that the SH3 domains of Crk-L and Crk II bound C3G. Since C3G activates Rap, a negative regulator of the Ras pathway, Crk proteins may participate in regulation of Ras signaling by the BCR.

Signaling by the B cell antigen receptor (BCR) plays an important role in both the establishment of immunologic tolerance and the generation of antibody (Ab) responses to foreign antigens. Immature B cells that bind self-antigens while still in the bone marrow are eliminated by apoptosis (1). In contrast, antigen binding by the BCR on newly formed mature B cells results in either activation, anergy, or apoptosis depending on the nature of the antigen and whether or not the B cell receives a co-stimulatory signal through other receptors such as CD40 (2, 3). In the presence of appropriate co-stimulatory signals and cytokines, BCR signaling promotes mature B cells to enter the cell cycle, proliferate, and differentiate into antibody-secreting cells (4).

To understand how BCR engagement regulates B cell survival and activation, it is necessary to elucidate the signaling pathways used by the BCR. Cross-linking of the BCR by multivalent antigens or by anti-immunoglobulin (Ig) Abs results in activation of several Src family tyrosine kinases as well as the Syk and Btk tyrosine kinases (5–7). These kinases then activate the signaling pathways that are controlled by phospholipase C-γ, phosphatidylinositol (3)kinase, and Ras (2). It is likely that the BCR also activates other signal transduction pathways that mediate the diverse effects of BCR engagement on B cells.

A common feature of many receptor signaling pathways is that the components of the pathway are physically separated in resting cells but are then assembled into signaling complexes after the receptor is engaged. In many cases, the assembly of these complexes is necessary to bring enzymes close to their substrates. This strategy promotes efficient signaling in receptor-activated cells while ensuring low basal levels of signaling in resting cells. Adapter proteins that contain SH2 and SH3 protein interaction domains play an important role in the assembly of signaling complexes. The SH3 domains of these proteins generally bind proteins in a constitutive manner, whereas the SH2 domains bind proteins that are tyrosine-phosphorylated in response to receptor signaling. In this way an adapter protein can inducibly co-localize proteins that bind to its SH2 and SH3 domains. The family of ubiquitously expressed SH2/SH3 adapter proteins includes Grb2, Crk, Nck, and the p85 subunit of PtdIns 3-kinase. Each of these proteins can bind a number of different signaling proteins via their SH2 and SH3 domains and may therefore participate in the formation of multiple signaling complexes.

We are interested in the role of SH2/SH3 adapter proteins in BCR signaling. We have previously shown that the Src and Grb2 adapter proteins may be involved in activation of Ras by the BCR. The SH3 domains of Grb2 bind SOS, a guanine nucleotide exchange factor that activates Ras by stimulating it to release GDP and bind GTP (8–10). In resting cells, the Grb2-SOS complex is cytoplasmic and is separated from Ras, which is tethered to the inner face of the plasma membrane. Receptor-induced phosphorylation of membrane proteins on appropriate tyrosine residues can create binding sites for the SH2 domain of Grb2 and recruit Grb2-SOS complexes to the plasma membrane, allowing SOS to activate Ras. For example, in fibroblasts stimulated with epidermal growth factor, the SH2 domain of Grb2 binds to phosphotyrosine-containing sequences in the cytoplasmic domain of the epidermal growth...
factor receptor (8, 11). In B cells, however, we found that the major target of the Grb2 SH2 domain is Shc, another cytoplasmic adapter protein that is tyrosine-phosphorylated after BCR cross-linking (12). The Shc SH2 domain can in turn bind to sites in the Ig-α/β subunit of the BCR, which are phosphorylated after BCR ligation (13, 14). Thus, Shc may allow the BCR to recruit Grb2/SOS complexes to the membrane where SOS can activate Ras.

The Crk adapter proteins may also be involved in regulating the Ras pathway. In addition to binding the Ras activator SOS, the N-terminal SH3 domain of Crk can bind C3G (15, 16). C3G is a nucleotide exchange factor that primarily activates another monomeric G protein called Rap (17). Rap competes with Ras for the same effectors and is thought to act as a negative regulator of Ras signaling pathways (18–20). Thus, Crk proteins could have either a positive or negative influence on Ras-mediated signaling. Crk proteins may also have additional roles in receptor signaling. A number of potential signaling proteins including the Ab1 tyrosine kinase (21), Eps15 (22), and DOCK180 (23) can associate with Crk.

Three different Crk proteins, termed Crk II, Crk I, and Crk-L, have been identified. It is not clear whether they are functionally identical. The 40/42-kDa Crk II protein has an N-terminal SH2 domain and two SH3 domains (Fig. 1). The 28-kDa Crk I protein is an alternatively spliced product of the crk II gene that lacks the C-terminal SH3 domain (24). The 38-kDa Crk-L protein is similar to Crk II, but is encoded by a separate gene (25). While the amino acid similarity between Crk II and Crk-L is only 60% overall, the SH2 and SH3 domains are highly conserved. This suggests that Crk II and Crk-L could interact with the same proteins, but this has not been analyzed extensively.

To determine if Crk proteins participate in BCR signaling, we asked whether BCR engagement caused the formation of signaling complexes involving either Crk-L or Crk II. In the RAMOS B cell line, we show that BCR ligation caused the SH2 domains of Crk-L and Crk II to bind to Cas and Cbl, two 120-kDa proteins that are tyrosine-phosphorylated in response to BCR engagement. Cas and Cbl contain multiple protein interaction motifs and may link Crk complexes with other signaling proteins. We also show that the SH3 domains of both Crk-L and Crk II bound primarily to C3G in RAMOS cells as opposed to SOS. Thus, Crk proteins may be involved in negative regulation of the Ras pathway in B cells. Finally, we show that BCR ligation increased the amount of Crk in the particulate fraction of RAMOS cells, suggesting that Crk proteins could move C3G from the cytosol to cellular membranes where Rap is located.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Glutathione S-Transferase (GST) Fusion Proteins**—Four different anti-Crk antibodies were used (Fig. 1): a rabbit polyclonal Ab raised against amino acids 283–302 of human Crk-L (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal Ab raised against amino acids 287–304 of human Crk II (Santa Cruz), the anti-Crk (102–304) monoclonal antibody (mAb), which was raised against amino acids 102–304 of human Crk II but recognizes both Crk II and Crk-L (Transduction Laboratories, Lexington, KY), and the 3A8 mAb raised against the SH2 domain of human Crk II (26). Rabbit Abs against Cbl and C3G were from Santa Cruz Biotechnology. The rabbit anti-Cas2 Ab has been described previously (27). mAbs against Grb2, SOS1/SOS2, and Cas were from Transduction Laboratories. The 4G10 anti-phosphotyrosine (anti-Tyr(P)) mAb was from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit anti-GST Ab was a gift from S. Robbins (University of Calgary). Rabbit IgG was purified from normal rabbit serum using protein A-Sepharose (Sigma).

GST fusion proteins containing the N-terminal SH3 domain of Crk (28), the SH2 domain of Crk (from T. Pawson, University of Toronto), full-length Grb2 (12), the SH2 domain of Grb2 (from D. Motto and G. Koretzky, University of Iowa) (29), or GST only (from S. Robbins, University of Calgary) were purified from bacterial lysates using glutathione-Sepharose 4B (Pharmacia, Baie d’Urfe, Quebec, Canada). The purity and integrity of the fusion proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining. Fusion protein concentrations were estimated by comparison to known amounts of bovine serum albumin (BSA) run on the same gel.

**Cell Culture and Stimulation**—The RAMOS human B lymphoma cell line was grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM t-glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. The cells were resuspended at 2.5 × 10^7/ml in modified Hapes-buffered saline (12) and stimulated with goat-anti-human IgM Abs (Bio-Can, Mississauga, Ontario, Canada) at a final concentration of 100 μg/ml. Reactions were stopped by adding cold phosphate-buffered saline containing 1 mM Na3VO4. After washing, the cells were solubilized at 5 × 10^7/ml in Triton X-100 lysis buffer (1% Triton X-100, 20 mM Tris–HCl, pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin). After 10 min on ice, detergent-insoluble material was removed by centrifugation. Cell lysates were stored at −80°C.

**Precipitation Experiments**—Cell lysate from 1.5 × 10^7 RAMOS cells was used for each precipitation. For immunoprecipitations, cell lysates were precleared for 30 min at 4°C with 10 μl protein A-Sepharose. Precleared cell lysates were mixed with Abs (1–2 μg) for 3 h at 4°C. Immune complexes were collected by adding 10 μl of protein A-Sepharose and mixing for an additional 1 h. When GST fusion proteins were used for precipitation, cell lysates were precleared for 1 h with 15 μl of glutathione-Sepharose and then mixed with 10 μg of fusion protein for 3 h. Fusion protein complexes were collected by adding 15 μl of glutathione-Sepharose and mixing for 1 h. When biotinylated peptides were used for precipitation, cell lysates were precleared for 1 h with 25 μl of avidin-agarose (Pierce). Precleared lysates were then mixed for 2 h with 25 μl of avidin-agarose beads to which 5 μg of biotinylated peptide had been adsorbed. In all cases, the beads were washed three times with PBS.
Crk Proteins Associate with Tyrosine-phosphorylated Proteins after BCR Ligation—SH2/SH3 adapter proteins assemble signaling complexes by using their SH2 domains to bind proteins that are tyrosine-phosphorylated in response to receptor engagement. Thus, if Crk proteins are involved in BCR signaling, BCR ligation should induce tyrosine phosphorylation of proteins that can bind to the Crk SH2 domain. To test this, we incubated lysates from the RAMOS B cell line with a GST fusion protein containing the SH2 domain of Crk II. We found that cross-linking the BCR on this cell line with anti-IgM Abs stimulated the tyrosine phosphorylation of several proteins that could bind to the Crk SH2 domain. This suggested that the Crk protein complexes in B cells could bind to the SH2 domains of Crk II and Crk-L and that Crk II after BCR ligation did so via the Crk SH2 domain fusion protein (Fig. 2A). It is likely that these phosphoproteins would also bind to the SH2 domain of Crk-L since the SH2 domains of Crk II and Crk-L are highly conserved.

Consistent with the in vitro results, we found that BCR cross-linking caused a similar set of tyrosine-phosphorylated proteins to bind to Crk-L and Crk II in RAMOS cells (Fig. 2, B and C). The 120-kDa and the 60-kDa Crk-associated phosphoproteins were always the most prominent. The Crk II phosphoprotein complexes were less abundant than the Crk-L phosphoprotein complexes (Fig. 2B), but could be readily observed with longer exposures (Fig. 2C). The simplest interpretation of this result is that Crk-L is expressed at higher levels than Crk II in RAMOS cells. The association of these tyrosine-phosphorylated proteins with Crk II was evident within 2 min of adding anti-IgM Abs to the RAMOS cells and persisted for at least 1 h (Fig. 2C). Further analysis showed that two 120-kDa phosphoproteins that associated with Crk-L and Crk II after BCR ligation did so via the Crk SH2 domain (see below).

In addition to causing Crk-L and Crk II to associate with several tyrosine-phosphorylated proteins, BCR ligation stimulated tyrosine phosphorylation of Crk-L and Crk II. When RAMOS cells were activated via their BCR, a 38-kDa tyrosine-phosphorylated protein was observed in anti-Crk-L immunoprecipitates (Fig. 2B), while a 40-kDa tyrosine-phosphorylated protein was seen in anti-Crk II immunoprecipitates (Fig. 2C). The molecular masses of these proteins suggested that they could be Crk-L and Crk II, respectively. Reprobing the blot in Fig. 2B showed that the 38-kDa tyrosine-phosphorylated protein in anti-Crk-L immunoprecipitates had the same electrophoretic mobility as Crk-L. Fig. 2C shows that BCR cross-linking caused some of the Crk II to migrate with a higher apparent molecular mass. Such bandshifts are often indicative of phosphorylation, and this higher molecular mass form of Crk II had the same electrophoretic mobility as the tyrosine-phosphorylated 40-kDa protein seen in anti-Crk II immunoprecipi-
and colleagues (27), while the 105-kDa protein may be an
likely to be the Cas protein that has been described by Hirai
Crk-L and to Crk II. The higher molecular mass protein is
cells, but BCR ligation significantly increased their binding to
TheseproteinswereassociatedwiththeCrkproteinsinresting
proteins of approximately 120 and 105 kDa that bound to Crk-L
C
sociated with Crk in activated RAMOS cells (see Fig. 2,
Cas was the 120-kDa tyrosine-phosphorylated protein that as-
formation causes substantial tyrosine phosphorylation of the
and Crk II after BCR cross-linking. In fibroblasts, v-
tyrosine-phosphorylated proteins that associated with Crk-L
induced the formation of both Crk-L
II-associated 120-kDa protein and to immunoprecipitated Cbl.

Indeed, the GST-Crk II SH2 fusion protein precipitated signif-
ificant amounts of Cbl from lysates of anti-IgM-stimulated
Cbl complexes and Crk-
Cbl complexes, with Crk-L
Cbl complexes, with Crk-L/Cbl complexes, with Crk-L/Cbl complexes being more preva-
ent (data not shown). While Crk-L and Crk II associated with
Cbl in a BCR-dependent manner, Grb2 associated constitu-
tively with Cbl in RAMOS cells (Fig. 4C). Immunoprecipitating with
Abs specific for either Crk-L or Crk II showed that BCR ligation
induced the formation of both Crk-L/Cbl complexes and Crk-
II/Cbl complexes, with Crk-L/Cbl complexes being more preval-
tant. While Crk-L and Crk II associated with Cbl in a BCR-dependent manner, Grb2 associated constitu-
tively with Cbl in RAMOS cells (Fig. 4D), pointing out a func-
tional difference between the Crk and Grb2 adapter proteins.

The inducible association of Cbl with Crk-L and Crk II sug-
ggested that Cbl binds to the SH2 domain of Crk proteins. Indeed, the GST-Crk II SH2 fusion protein precipitated signif-
ificant amounts of Cbl from lysates of anti-IgM-stimulated
RAMOS cells but very little Cbl from lysates of unstimulated
cells (Fig. 5A). While a small amount of Cbl from both stimu-
lated and unstimulated RAMOS cells bound to the GST-Crk
N-terminal SH3 domain fusion protein, Cbl bound primarily to
the Crk SH2 domain and its ability to do so correlated with its
phosphorylation on tyrosine residues.

To determine whether Cbl bound directly to the SH2 domain
of Crk proteins, we used the GST-Crk SH2 domain fusion protein to probe blots of anti-Crk and anti-Cbl immunoprecipi-
tates (Fig. 5B). The Crk SH2 domain bound directly to the Crk
II-associated 120-kDa protein and to immunoprecipitated Cbl.

BCR ligation increased the ability of the GST-Crk SH2 domain
alternatively spliced form of Cas or a Cas-related protein. Unless otherwise indicated, we will refer to them collectively as
Cas.

To determine how Cas interacted with Crk proteins, we
incubated RAMOS cell lysates with GST fusion proteins con-
taining either the Crk II SH2 domain or the Crk II N-terminal
SH3 domain (Fig. 3B). Both forms of Cas bound specifically to
the Crk SH2 domain but not to the Crk SH3 domain. Moreover, the Cas proteins did not bind to a GST-Grb2 SH2 domain fusion protein. The Crk II SH2 fusion protein precipitated a small amount of Cas from unstimulated RAMOS cells but much more from anti-IgM-stimulated cells. This suggested that
tyrosine residues in Cas that mediate binding to Crk proteins
were phosphorylated at low levels in unstimulated RAMOS
cells and that the phosphorylation of these residues was in-
creased by BCR ligation. Consistent with this idea, anti-Tyr(P)
immunoblotting showed that BCR ligation stimulated tyrosine
phosphorylation of the 120-kDa Cas protein in RAMOS cells
(Fig. 3C). We were unable to detect tyrosine phosphorylation of
the p105 form of Cas. The rabbit anti-Cas Ab used for immu-
noprecipitation does not recognize p105 Cas as well as p120
Cas and may not recognize the phosphorylated form of p105
Cas. Nevertheless, these data clearly show that BCR ligation
induces Crk proteins to bind to p120 Cas and a 105-kDa protein
that may be related to Cas.

**Cbl Is Tyrosine-phosphorylated and Binds Directly to the Crk SH2 Domain**—Another candidate tyrosine-phosphorylated
protein that could bind to the Crk SH2 domain in B cells is the
120-kDa Cbl protein. Cbl is tyrosine-phosphorylated in re-
response to BCR cross-linking (30, 31) and has been shown to
bind to Crk proteins in activated T cells (32, 33). While Crk-L and Crk II associated with
Cbl complexes, with Crk-L/Cbl complexes, with Crk-L/Cbl complexes being more preva-
lent (data not shown). While Crk-L and Crk II associated with
Cbl in a BCR-dependent manner, Grb2 associated constitu-
tively with Cbl in RAMOS cells (Fig. 4C). Immunoprecipitating with
Abs specific for either Crk-L or Crk II showed that BCR ligation
induced the formation of both Crk-L/Cbl complexes and Crk-
II/Cbl complexes, with Crk-L/Cbl complexes being more prevalent.

Fig. 3. Crk inducibly associates with Cas. RAMOS cells were incubated for 2 min with (+) or without (−) anti-IgM Abs. A, cell lysates were precipitated with the anti-Crk-L Ab, the anti-Crk II polyclonal Ab, or with rabbit IgG (control). Precipitated proteins were analyzed by blotting with an anti-Cas mAb. Molecular mass standards (in kDa) are indicated to the left. B, cell lysates were precipitated with the indicated fusion proteins or with the anti-Cas mAb. Precipitated proteins were analyzed by blotting with the anti-Cas mAb. C, cell lysates were pre-
precipitated with the rabbit anti-Cas2 Ab or with a control Ab. Precipitated proteins were analyzed by anti-Tyr(P) (Anti-P-Tyr) immunoblotting. The filter was then stripped and reprobed with the anti-Cas mAb.

tates from activated RAMOS cells. Thus, it appears that both
Crk-L and Crk II are tyrosine-phosphorylated in response to
BCR ligation. This may allow other SH2-containing proteins to
bind to Crk-L or Crk II.

Cas Inducibly Associates with Crk Proteins—To elucidate the
role of Crk proteins in BCR signaling, we tried to identify the
tyrosine-phosphorylated proteins that associated with Crk-L
and Crk II after BCR cross-linking. In fibroblasts, v-src transforma-
tion causes substantial tyrosine phosphorylation of the
120–130-kDa Cas protein, creating multiple sites to which the
Crk SH2 domain can bind (27). Therefore, we asked whether
Cas was the 120-kDa tyrosine-phosphorylated protein that asso-
ciated with Crk in activated RAMOS cells (see Fig. 2, B and
C). Immunoblotting with an anti-Cas mAb revealed two pro-
teins of approximately 120 and 105 kDa that bound to Crk-L
and Crk II in anti-IgM-stimulated RAMOS cells (Fig. 3A). These proteins were associated with the Crk proteins in resting
cells, but BCR ligation significantly increased their binding to
Crk-L and to Crk II. The higher molecular mass protein is
likely to be the Cas protein that has been described by Hirai
and colleagues (27), while the 105-kDa protein may be an

2 H. Hirai, unpublished observations.
Cbl is tyrosine-phosphorylated and associates with Crk after BCR cross-linking. RAMOS cells were incubated for 2 min with (+) or without (−) anti-IgM Abs. A, cell lysates were precipitated with the anti-Crk II Ab, with the anti-Cbl Ab, or with rabbit IgG (control). Precipitated proteins were analyzed by anti-Tyr(P) (Anti-P-Tyr) immunoblotting. Molecular mass standards (in kDa) are indicated to the left. B, cell lysates were precipitated with the anti-Crk (102–304) mAb or with an isotype-matched mAb (control) and analyzed by immunoblotting with an anti-Cbl Ab. C, cell lysates were precipitated with the anti-Cbl Ab or with rabbit IgG (control) and analyzed by immunoblotting with the anti-Crk (102–304) mAb. D, cell lysates were precipitated with the anti-Cbl Ab or with rabbit IgG (control) and analyzed by immunoblotting with an anti-Grb2 mAb. Cell lysate from 5 × 10⁵ cells was included as a positive control.

Fusion proteins to bind to immunoprecipitated Cbl. The simplest interpretation of these data is that BCR-induced tyrosine phosphorylation of Cbl creates binding sites for the SH2 domain of Crk proteins. Thus, BCR ligation caused Crk proteins to bind via their SH2 domains to two different 120-kDa proteins, Cas and Cbl.

Although Crk II binds to Shc in PC12 cells (16), we found that Crk-L and Crk II did not bind to Shc in activated RAMOS cells (data not shown). This is in contrast to Grb2, whose SH2 domain binds primarily to phosphorylated Shc in B cells (12). Thus, the SH2 domains of Crk and Grb2 have different targets in activated B cells. This suggests that proteins that bind to the SH3 domains of Crk and Grb2 can be directed to different cellular locations and can interact with different proteins.

Crk and Grb2 Associate with Different Exchange Factors in RAMOS B Cells—The co-localization of proteins that bind to the SH2 and SH3 domains of an adapter protein may be required for efficient signal transduction. Having shown that BCR ligation causes Cas and Cbl to bind to the SH2 domains of Crk-L and Crk II, it was important to characterize the proteins that bind to the SH3 domains of Crk-L and Crk II in B cells. Crk proteins have been shown to associate with two nucleotide exchange factors, SOS and C3G (15, 16). SOS is an activator of Ras (9, 10), while C3G activates Rap (17), a G protein that acts as a negative regulator of the Ras pathway by competing for the same effectors as Ras (18–20). Thus, Ras-mediated signaling may reflect a balance between the actions of SOS and C3G.

In addition to binding Crk proteins, SOS and C3G can also associate with Grb2 (8, 15). While this suggests that Crk and Grb2 could have similar roles in regulating the Ras pathway, it is not known whether Crk-SOS, Crk-C3G, Grb2-SOS, and Grb2-C3G complexes are all present in significant amounts in B cells. To determine which adapter protein-exchange factor complexes are likely to be most prevalent in B cells, we first assessed the ability of SOS and C3G to bind GST fusion proteins containing either the Crk II N-terminal SH3 domain or the entire Grb2 protein. We found that SOS bound equally well to the Crk II SH3 and Grb2 fusion proteins in vitro (Fig. 6A). The anti-SOS mAb we used for immunoblotting recognizes both human SOS1 and SOS2. In contrast to SOS, C3G bound much better to the Crk II SH3 domain than to Grb2.

We then investigated whether the relative abilities of SOS and C3G to bind Grb2 in vitro reflected which complexes were present in the RAMOS B cell line. Crk-L, Crk II, and Grb2 were precipitated from cell lysates and the precipitates were probed with Abs to SOS or C3G (Fig. 6, B–D). In these experiments, Crk II was specifically precipitated with the 3A8 mAb, which recognizes an epitope in the SH2 domain of Crk II that is not present in Crk-L (26). Unlike other anti-Crk II Abs that bind epitopes near the SH3 domains, the 3A8 mAb can precipitate Crk II with proteins bound to its SH3 domains. Since the binding of proteins to the SH3 domains of Grb2 can also block Ab binding, we precipitated Grb2 with a phosphotyrosine-containing peptide (ELFDDPSpYVNQNLKD; single-letter code, pY = phosphotyrosine) based on the sequence in Shc that binds to the Grb2 SH2 domain. This peptide precipitated a substantial portion of the Grb2 in RAMOS cells (data not shown).

Consistent with the fusion protein experiments, the C3G in
Fig. 6. Association of SOS and C3G with Crk and Grb2. A, RAMOS cell lysates were precipitated with the indicated fusion proteins. Precipitated proteins were analyzed by immunoblotting with the anti-C3G Ab or the BCR with anti-IgM Abs (Fig. 6B). The bands were stripped and reprobed with a mAb that recognizes both SOS1 and SOS2. Cell lysate from 5 × 10⁶ cells was included as a positive control. Molecular mass standards (in kDa) are indicated to the left. B, cell lysates were precipitated with the indicated peptides immobilized on beads, with the 3A8 anti-Crk II mAb, or with an isotype-matched control mAb. The Shc Tyr(P) (P-Tyr) peptide (ELF-DDPSpYVNVQNLKD) is based on the sequence in Shc that binds to the Crk-L SH2 domain. The non-phosphorylated version of this peptide, as well as an irrelevant Tyr(P)-containing peptide (LQSDpYMNMTP), neither of which precipitated Grb2 (data not shown), were used as controls. Sequential immunoblotting with the anti-C3G Ab and then with the anti-SOS mAb was performed as in A. Cell lysate from 5 × 10⁶ cells was included as a positive control. Note that the anti-SOS Ab reacted with a band in the 3A8 immunoprecipitates from RAMOS cells (lane marked R). However, this band had a different electrophoretic mobility than SOS and is likely to be a contaminant in the Ab preparation since it was present when the cell lysate was omitted from the reaction and replaced with Triton X-100 lysis buffer (lane marked LB). C, cell lysates were precipitated with the Shc Tyr(P) peptide, with a Crk-L-specific Ab, or with rabbit IgG (control). Sequential immunoblotting with the anti-C3G Ab and then with the anti-SOS mAb was performed as in A. Cell lysate from 5 × 10⁶ cells was included as a positive control. D, RAMOS cells were incubated for 2 min with (+) or without (−) anti-IgM Abs. Cell lysates were precipitated with the indicated Abs or with purified rabbit IgG (control) and immunoblotted with the anti-C3G Ab. Cell lysate from 5 × 10⁶ cells was included as a positive control.

RAMOS cells preferentially associated with Crk as opposed to Grb2. C3G was precipitated by the 3A8 anti-Crk II mAb (Fig. 6B) and by the anti-Crk-L Ab (Fig. 6C) but not by the Shc phosphopeptide that precipitates Grb2 (Fig. 6, B and C). More C3G associated with Crk-L than with Crk II (Fig. 6D), consistent with the idea that Crk-L is more abundant than Crk II in RAMOS cells. The interaction of C3G with Crk-L and Crk II was evident in unstimulated RAMOS cells and did not change upon ligation of the BCR with anti-IgM Abs (Fig. 6D).

Although SOS bound the SH3 domains of Crk and Grb2 equally well in vitro, it preferentially bound to Grb2 in RAMOS cells. Much less SOS was bound to Crk-L than to Grb2 (Fig. 6C) and SOS could not be detected in anti-Crk II immunoprecipitates (Fig. 6B). The weak binding of SOS to Crk proteins in vivo may reflect the ability of C3G to compete more effectively for binding to Crk. C3G may have higher affinity for the Crk SH3 domain than SOS (34), or it may be expressed at higher levels than SOS1 and SOS2. Thus, C3G binds exclusively to the Crk proteins in RAMOS cells, while SOS associates primarily with Grb2 and to a lesser extent with Crk-L.

Subcellular Localization of Crk, Cas, and Cbl—Both Crk and C3G are cytosolic proteins, whereas Rap is targeted to the cytosolic face of cellular membranes by a lipid modification (35, 36). The ability of Crk-C3G complexes to regulate Rap may therefore require translocation of these complexes from the cytoplasm to cellular membranes. To see if this occurred in RAMOS cells, we analyzed the subcellular localization of the Crk proteins before and after BCR ligation. Immunoblotting the soluble and particulate fractions of RAMOS cells with the anti-Crk (102–304) mAb showed that the majority of the Crk proteins were in the soluble fraction (Fig. 7A). While a small amount of Crk was present in the particulate fraction of unstimulated cells, BCR ligation increased the amount of Crk proteins in the particulate fraction. This suggests that BCR signaling caused Crk proteins to translocate from the cytosol to cellular membranes.

Since the Crk SH2 domain binds to Cas and Cbl, we asked...
whether Cas and Cbl were in the particulate fraction of RAMOS cells. Immunoblotting with anti-Cas Abs (Fig. 7B) or with anti-Cbl Abs (Fig. 7C) showed that the majority of these proteins were in the soluble fraction of RAMOS cells. However, significant amounts of Cas and Cbl were present in the particulate fractions of both unstimulated and anti-IgM-stimulated RAMOS cells. BCR ligation did not significantly alter the subcellular distribution of Cas or Cbl. To confirm that the particulate fraction was not contaminated with cytoplasmic proteins, we showed that virtually all of the Vav protein was in the soluble fraction (data not shown). Thus, small but significant amounts of Cas and Cbl were in the particulate fraction of RAMOS cells, even before BCR ligation.

Tyrosine phosphorylation of membrane-associated Cas and Cbl could provide binding sites for the Crk SH2 domain. This would allow Crk proteins to bring C3G and other proteins that bind to their SH3 domains to the membrane. To test this model, we asked whether Crk-Cas complexes or Crk-Cbl complexes could be found in the particulate fraction of RAMOS cells. We found that BCR ligation increased the amount of Crk-L-Cas complexes in both the particulate and soluble fractions of RAMOS cells (Fig. 8A). Approximately 50% of the Crk-L-Cas complexes were in the particulate fraction of anti-IgM-stimulated RAMOS cells. Similarly, BCR cross-linking caused a large increase in the amount of Crk-L-Cbl complexes in the particulate fraction of RAMOS cells (Fig. 8B). Crk-L-Cbl complexes were also found in the soluble fraction of RAMOS cells. We were not able to detect membrane-associated Crk II-Cas complexes or Crk II-Cbl complexes, presumably because Crk II is expressed at lower levels than Crk-L. Nevertheless, our data show that BCR cross-linking induced the formation of membrane-associated Crk-L-Cas complexes and Crk-L-Cbl complexes in RAMOS cells.

DISCUSSION

We have made several novel observations concerning the role of the Crk adapter proteins in BCR signaling. We provide the first evidence that both Crk-L and Crk II are tyrosine-phosphorylated in response to BCR ligation, and we show that several tyrosine-phosphorylated proteins associate with the Crk proteins after BCR cross-linking. We identified two of these phosphoproteins as Cas and Cbl and showed that both bound to the SH2 domains of Crk-L and Crk II after BCR engagement. This is the first report that Cas is a target of BCR-associated tyrosine kinases. We also show that in the RAMOS B cell line the SH3 domains of both Crk-L and Crk II preferentially bind the C3G nucleotide exchange factor as opposed to SOS. Since C3G activates Rap, it suggests that Crk proteins are involved in negative regulation of Ras-mediated signaling in B cells. Our cell fractionation studies showed that Cas and Cbl are present to some extent in the particulate fraction of RAMOS cells and may therefore provide docking sites that can recruit Crk complexes to cellular membranes. Consistent with this idea, we found that BCR ligation increased the amount of Crk in the particulate fraction of RAMOS cells and induced the formation of Crk-L-Cas and Crk-L-Cbl complexes in the particulate fraction. This is the first report suggesting that Crk proteins move from the cytoplasm to cellular membranes in response to receptor signaling. Crk-mediated translocation to cellular membranes may be important for C3G to activate Rap and for other Crk-associated proteins to perform their functions.

We have identified Cas and Cbl as two major targets of the Crk SH2 domain in activated B cells. Smit et al. (37) have also shown that Cbl binds to Crk proteins after BCR cross-linking. Cas and Cbl can be considered part of another family of adapter proteins that includes IRS-1, IRS-2, Gab1, and the Drosophila DOS protein. These proteins contain various protein interaction motifs and are also phosphorylated on multiple tyrosine residues that serve as docking sites for SH2 domain-containing proteins. Cas has an SH3 domain as well as 15 YXXP (single-letter code; X is any amino acid) motifs that could bind the SH2 domains of Crk or Nck (27). Similarly, Cbl has 17 proline-rich motifs that could potentially bind SH3 domains (38) and is also strongly phosphorylated on tyrosine residues in response to BCR ligation (30, 31). Thus, multiple signaling proteins could simultaneously bind a single molecule of Cas or Cbl. In B cells, Cbl binds Grb2 and PtdIns 3-kinase (31, 39) in addition to Crk. This may allow cross-talk between Crk-associated proteins and signaling pathways involving Grb2 and PtdIns 3-kinase.

Cas and Cbl may also link tyrosine kinases to Crk and Crk-associated proteins. In B cells, Cbl associates with the Fyn and Btk tyrosine kinases (30, 39). Cas has also been reported to bind Src kinases (27). These tyrosine kinases could phosphorylate the Crk proteins as well as proteins bound to the Crk SH3 domains. Tyrosine phosphorylation of Crk-L and Crk II could allow SH2-containing proteins to bind to them and may provide another means by which Crk proteins can mediate the formation of signaling complexes.

In RAMOS cells, we found that both Crk-L and Crk II bound C3G via their SH3 domains. Smit et al. (37) recently reported that Crk-L binds C3G in RAMOS cells but Crk II does not. The Ab they used to precipitate Crk II recognizes an epitope in the N-terminal SH3 domain of Crk II (see Fig. 1). We found that this Ab did not precipitate Crk II-C3G complexes, presumably because this epitope on Crk II is masked by the binding of C3G. However, when we used the 3A5 anti-Crk II mAb, which recognizes an epitope in the SH2 domain of Crk II (but not Crk-L), we were able to clearly show that Crk II does bind C3G in RAMOS cells. Thus, Crk-L and Crk II are likely to have similar functions in B cells.

C3G is a nucleotide exchange factor that activates the Rap1A and Rap1B G proteins (17). We are currently testing whether BCR ligation activates Rap proteins in B cells. Receptor-in-
duced Rap activation has not been reported in any system. Rap may be a key signaling molecule since loss-of-function mutations in Drosophila Rap1 are lethal (20). While Rap may participate in multiple signaling pathways, several studies have shown that Rap1A is a negative regulator of the Ras signaling pathway. Overexpression of Rap1A inhibits fibroblast transformation by oncogenic versions of Ras (40) and blocks Ras-dependent germinal vesicle breakdown in Xenopus oocytes (41). Similarly, a gain-of-function mutation in the Drosophila rap1 gene blocks Ras-dependent development of the R7 photoreceptor cell (20). Activated Rap1A does not prevent Ras activation (19) but instead blocks the ability of Ras to interact with and activate downstream effectors. Rap has the identical effector-interaction sequence as Ras (18), and this allows Rap-GTP to compete with Ras-GTP for binding to Ras effectors. Rap-GTP is thought to sequester Ras effectors and prevent them from being activated by Ras. Potential downstream effectors of Ras include the Raf-1 kinase (42), PtdIns 3-kinase (43), the Ras GTPase-activating protein (44), and Ral-GDS, a nucleotide exchange factor that activates another member of the Ras family called Ral (45, 46). Rap-GTP can bind all of these proteins (46–49) and could potentially inhibit all Ras-mediated signaling events.

The competition between Ras-GTP and Rap-GTP for binding to Raf-1 may be of particular significance. The binding of Ras-GTP to Raf-1 initiates a protein kinase cascade that culminates in activation of the ERK (extracellular signal-regulated kinase) family of mitogen-activated protein kinases. The ERKs are important regulators of cell growth and differentiation that phosphorylate and activate transcription factors such as Elk-1 (50). Activation of the Ras/Raf/ERK pathway by the BCR and other receptors is usually transient. For example, BCR-induced activation of Raf-1 is maximal after 1 min and declines to near basal levels by 15 min (51). Prolonged Ras signaling may be deleterious to cells and this may be prevented by activation of Rap, which can then sequester Raf effectors such as Raf-1. Overexpression of constitutively active Rap1A in fibroblasts has been shown to block Ras-dependent activation of ERKs by epidermal growth factor (19). Whether Rap1A normally limits the magnitude or duration of ERK activation in B cells or other cells remains to be determined.

The regulation of downstream effectors of Ras (e.g., Raf-1) may involve a balance between SOS-mediated activation of Ras and C3G-mediated activation of Rap. Our data suggest that in B cells the Crk and Grb2 adapter proteins have opposing functions in this process since they preferentially bind different nucleotide exchange factors (Fig. 9A). In Ramos cells, Grb2 bound SOS but did not bind detectable amounts of C3G. Thus, Shc and Grb2 may promote BCR-induced Ras activation. Crk-L/SOS complexes may also make a minor contribution to BCR-induced Ras activation. However, we found that the Crk proteins associate primarily with C3G in Ramos cells and could therefore be involved in Rap-mediated down-regulation of Ras signaling pathways.

In addition to C3G, Crk proteins may control the interactions and subcellular localization of other proteins that bind to their SH3 domains. The Crk N-terminal SH3 domain can bind the Abl tyrosine kinase (21), two tyrosine kinase substrates of unknown function called EPS15 and EPS15R (22), and an SH3-containing protein called Dock180 whose function is also unknown (23). It is not known whether these proteins associate with Crk proteins in B cells. Preliminary experiments have shown that the 60-kDa tyrosine-phosphorylated protein that associates with Crk-L and Crk II in Ramos cells (Fig. 2, B and

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**Fig. 9. Proposed role of Crk proteins in BCR signaling.** A, positive and negative regulation of Ras-mediated signaling by Grb2/SOS complexes and Crk/C3G complexes. B, recruitment of Crk/C3G complexes to cellular membranes by binding to Cas and Cbl. C, Crk binds to the Crk N-terminal SH3 domain. We are currently investigating whether this 60-kDa protein is a novel protein or if it is related to proteins of similar molecular masses that associate with Ras GTPase-activating protein, Grb2, and PtdIns 3-kinase.

Our cell fractionation studies showed that BCR ligation induced the appearance of Crk-L/Cas and Crk-L/Cbl complexes in the membrane-enriched particulate fraction of Ramos cells. This correlated with an increase in the amount of Crk proteins in the particulate fraction. In contrast, a similar amount of Cas and Cbl were present in the particulate fraction before and after BCR ligation. This suggests a model in which BCR-induced tyrosine phosphorylation of membrane-associated Cas and Cbl creates binding sites for the Crk SH2 domain and thereby recruits Crk proteins to cellular membranes (Fig. 9B). While it is not clear how Cas and Cbl associate with membranes, it may be due to their ability to bind Src kinases such as Fyn, which are anchored to cellular membranes by lipid modifications. The significance of BCR-induced translocation of Crk protein complexes to cellular membranes remains to be determined. It may be critical for C3G to activate Rap. Microscopy studies will be required to determine which cellular membranes Rap is associated with in B cells and whether Crk/C3G complexes translocate to those membranes after BCR ligation.

In summary, we have shown that the Crk-L and Crk II adapter proteins are used by the BCR to promote the formation of signaling protein complexes. The assembly of these complexes may initiate signaling reactions by co-localizing components of signaling pathways and allowing efficient signal transmission.

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3 R. J. Ingham, C. Siu, and M. R. Gold, unpublished observations.
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