Syk-dependent Phosphorylation of Shc

A POTENTIAL LINK BETWEEN FcRI AND THE Ras/MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING PATHWAY THROUGH SOS AND Grb2

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Bana J abril-Cuenod†, Cheng Zhang§, Andrew M. Scharenberg¶, Rossella Paolinit†, Robert Numeroft, Michael A. Beaven§, and Jean-Pierre Kinet‡†

From the Molecular Allergy and Immunology Section, National Institute of Allergy and Infectious Diseases and the Laboratory of Molecular Immunology, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

Antigen receptors on T- and B-cells activate Ras through a signaling pathway that results in the tyrosine phosphorylation of Shc and the formation of a complex of Shc with the Grb2 adaptor protein. The high affinity receptor for immunoglobulin E (FcεRI) in cultured mast (RBL-2H3) cells has been reported to function differently. Here we show to the contrary that engagement of FcεRI with antigen leads to increased tyrosine phosphorylation of Shc and the association of Shc with Grb2 and other proteins (p120 and p140). Like the FcεRI-mediated activation of the mitogen-activated protein kinase cascade, these responses are dependent on the tyrosine kinase Syk; they are enhanced by overexpression of Syk and are blocked by expression of dominant-negative Syk. Shc is constitutively associated with Grb2 in these cells but dissociates from Shc on stimulation with antigen. These reactions are rapid, reversible, and associated with the activation of Ras. Therefore, the Syk-dependent tyrosine phosphorylation of Shc and its association with Grb2 may provide a pathway through SOS for activation of Ras by FcεRI.

Tyrosine kinase-dependent receptors, such as the EGF receptor, utilize an intermediate protein, Shc, the adaptor protein, Grb2, and the guanine nucleotide exchange factor, SOS, to convert Ras (p21GTP) to its active GTP-bound state (1–4) which, in turn, initiates signals for cell growth and differentiation via MAP kinase and other pathways (5). Two scenarios are possible. In one, SOS, which is constitutively bound to the SH3 domain of Grb2 (6–9), is translocated to the membrane by binding of the SH2 domains of Grb2 to tyrosine-phosphorylated motifs of activated EGF receptors (6, 10). This translocation does not enhance the exchange activity of SOS (7, 11), but it is thought, instead, to allow SOS to interact with membrane-bound Ras because Ras can be activated by fusing SOS directly with the plasma membrane (12, 13).

In the second scenario, the Grb2-SOS complex binds to receptor-bound phosphorylated Shc (13, 14). Shc is tyrosine-phosphorylated after binding, via its SH2 domain, to the phosphorylated motifs of the activated EGF receptor (1). The formation of the Shc-Grb2-SOS complex correlates with the formation of the GTP-bound state of Ras (15), but other reactions take place. These include formation of ternary complexes of phosphorylated Shc-Grb2 with other proteins, namely p120 and p140 (16, 17) and the phosphorylation of SOS by protein kinase C (18) and MAP kinase (19, 20) with an associated retardation in the electrophoretic mobility of SOS (20, 21). It is unclear whether the above two scenarios provide alternate or redundant mechanisms for activation of Ras.

The multimeric B-cell and T-cell antigen receptors appear to utilize similar mechanisms for the sustained activation of the p21MAP kinase signaling pathway. These receptors, unlike growth factor receptors, recruit the cytosolic tyrosine kinases, Src and Syk/ZAP70, which bind to tyrosine-phosphorylated motifs (ITAMs) that are present in the cytosolic tail of various subunits of these receptors (22). The subsequent activation of these kinases results in tyrosine phosphorylation of cellular proteins including Shc, formation of phosphorylated Shc-Grb2-SOS complexes (23, 24), and the activation of the MAP kinase pathway via Ras (4).

Analogous to the T- and B-cell antigen receptors, antigen-induced aggregation of the mast cell FcεRI, the multimeric high affinity receptor for IgE, leads to tyrosine kinase-dependent activation of the MAP kinase pathway (25), but the pathways leading to the activation of Ras and the activation of Ras itself have not been established. The β and γ chains of FcεRI contain ITAMs (22) that allow recruitment of the tyrosine kinases, Lyn and Syk (26–32), and the tyrosine phosphorylation of various proteins (33–35), including FcεRI β and γ chains (28, 30, 31, 36) and Vav (25) which has been implicated in the activation of Ras in B- and T-cells (37, 38). Expression of porcine Syk, or a dominant-negative truncated Syk (Syk-T) that lacks the kinase domain (25, 32), in a vaccinia expression system, has indicated that Syk is essential for activation of the MAP kinase-phosphatidylinositol 3-kinase/AKT pathway in cultured RBL-2H3 mast cells (25). This activation is apparent from an increase in MAP kinase activity (25, 39, 40) and the tyrosine phosphorylation and shift in electrophoretic migration of p42MAPK (41).

As reported here, we have established that Ras and the Shc-Grb2-SOS pathway are activated in RBL-2H3 cells via FcεRI and that the constitutive phosphorylation of Shc, as noted by others (17), could be minimized by reducing the serum content of the medium. Furthermore, overexpression of active or dominant negative forms of Syk demonstrate that tyrosine...
phosphorylation of Shc and its association with Grb2 and the proteins p120 (possibly c-Cbl) and p140 are dependent on Syk. The studies provide the first definitive indication of communication between FcεRI and the Ras/MAP kinase cascade through Shc, Grb2, and Sos via Syk.

**Materials and Methods**

Reagents—These were obtained from the following sources: sera and media from Biofluids (Rockville, MD) and Life Technologies, Inc.; dinitrophenyl-human serum albumin (catalog number A666), β-dinitrophenyl-lysine, phenyl phosphate, GTP and GDP from Sigma; [32P]orthophosphate and Enhanced Chemiluminescent detection kit from Amersham Corp.; Protein A-Sepharose beads from Pharmacia Biotech Inc.; mini-gels from Novex Experimental Technologies (San Diego, CA); and PEI-cellulose plates from EM Separation (Gibbstown, NJ); anti-phosphotyrosine 4G10, anti-Shc, which recognizes the p46 isoform of Shc, and anti-Sos antibodies from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-Grb2 antibody from Transduction Laboratories (Lexington, KY) and Santa Cruz (Santa Cruz, CA); rabbit anti-rat antibody from Jackson Immunoresearch (West Grove, PA); rat anti-Ras antibody (Ha-RasV259) and rabbit anti-c-Cbl antibody from Transduction Laboratories (Lexington, KY) and Santa Cruz (Santa Cruz, CA); rabbit anti-phosphotyrosine 4G10, anti-Shc, which recognizes the p46 isoform of Shc, and anti-Sos antibodies from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-Grb2 antibody from Transduction Laboratories (Lexington, KY) and Santa Cruz (Santa Cruz, CA); rabbit anti-rat antibody from Jackson Immunoresearch (West Grove, PA); rat anti-Ras antibody (Ha-RasV259) and rabbit anti-c-Cbl antibody from Santa Cruz. Rabbit polyclonal anti-porcine Syk (42) and monoclonal anti-DNP IgE (32) were prepared as described.

**Cell Culture and Infection with Vaccinia Expression System—**RBL-2H3 cells were maintained in monolayer culture in minimal essential medium supplemented with Earle's salts, 15% heat-inactivated fetal bovine serum, and 1% L-glutamine at 37°C in 5% CO2. Recombinant vaccinia viruses were prepared, and infection of RBL-2H3 cells was performed as described previously (25, 32). Positions of GDP and GTP standards are indicated. B, cells were stimulated for the indicated times for separation of Ras-bound GDP and GTP as described above. The amounts of radioactive nucleotides were determined by scanning plates for β emission. Data are expressed as percentage of radioactive GTP recovered divided by total radioactive nucleotides recovered. Data are from one of three similar experiments.

**Activation of Ras, Tyrosine Phosphorylation of Shc, and Association of Grb2 with Phosphorylated Shc and Sos—**As shown in Fig. 1, stimulation of RBL-2H3 cells for 1 min with the antigen, DNP-BSA, resulted in an increase in GDP-bond Ras (panel A). This increase was apparent within 15 s, reached a maximum by 1 min, and was sustained for at least 10 min (panel B).

**Results and Discussion**

Activation of Ras, Tyrosine Phosphorylation of Shc, and Association of Grb2 with Phosphorylated Shc and Sos—As shown in Fig. 1, stimulation of RBL-2H3 cells for 1 min with the antigen, DNP-BSA, resulted in an increase in GDP-bound Ras (panel A). This increase was apparent within 15 s, reached a maximum by 1 min, and was sustained for at least 10 min (panel B).
Activation of the Shc-Grb2-Sos Pathway via FcεRI and Syk

size corresponded to p46shc and p52shc (p56shc was not detected) (1, 44). Of these two forms, p52shc was most heavily phosphorylated (Fig. 2A, lane 6). Increased phosphorylation of two additional proteins (estimated sizes, 120 and 140 kDa), which co-immunoprecipitated with Shc, was also apparent (see Fig. 2A, lanes 4 and 5). Subsequent addition of excess monovalent hapten, DNP-lysine, caused rapid reversal in the state of phosphorylation of Shc (Fig. 2B) and p120 and p140 (not shown).

Consistent with the 32P-labeling experiments, the stimulation with antigen resulted in increased tyrosine phosphorylation of Shc as indicated by anti-phosphotyrosine blots of the Shc immunoprecipitates (Fig. 2C). In this and four other experiments, p52shc was predominantly tyrosine-phosphorylated. In general the tyrosine phosphorylation of p46shc was weaker but variable. Reprobing of blots with anti-Shc antibody confirmed the identity of Shc and that the total amount of Shc protein was unchanged. Immunoblotting with anti-Grb2 antibody also revealed an increased association with Grb2 with Shc (Fig. 2D).

Grb2 also co-immunoprecipitated with Sos, but this association was apparent in both unstimulated and stimulated cells (Fig. 2E). Although the association of Sos with Grb2 was enhanced in some experiments, in others the enhancement was minimal (as in Fig. 2E). Sos was also constitutively associated with Shc, as indicated by co-immunoprecipitation of Sos with Shc, but appeared to dissociate from Shc in stimulated cells (compare stimulated cells, lane 2, with unstimulated cells, lanes 1 and 3, in Fig. 2F). A small shift in migration of Sos was also apparent in these gels. A possible explanation would be the phosphorylation of Sos by either protein kinase C or MAP kinase (see Introduction).

Attempts were made to identify the p120 and p140 proteins. A likely candidate for p120 was the proto-oncogene product, c-Cbl, which was shown to be a substrate for receptor-activated tyrosine kinases in B- and T-cells and to associate with the SH3 domain of Grb2 and other proteins (45–47). In the Shc immunoprecipitates, phosphorylated p120 was found to co-migrate with c-Cbl. Immunoprecipitation of c-Cbl indicated that it was tyrosine-phosphorylated upon cell stimulation and that it co-precipitated tyrosine-phosphorylated p140 (data not shown). Thus, c-Cbl might associate with both Shc and p140; the identity of p140 and the relevance of this association are under investigation.

Dependence of Events on Syk as Demonstrated by Expression of Syk-T and Porcine Syk—In cells infected with wild type vaccinia virus and then labeled with [32P]orthophosphate, 32P-phosphorylation of p56shc was still apparent in antigen-stimulated cells (Fig. 3A, compare lane 2 with 1). This phosphorylation was enhanced in cells that were infected with Syk-recombinant virus (Fig. 3A, lanes 3 and 4) but blocked in cells infected with Syk-T-recombinant virus (Fig. 3A, lanes 5 and 6). In a matching experiment both p46shc and p52shc were tyrosine-phosphorylated in cells infected with either wild type or Syk-recombinant virus (Fig. 3B, lanes 1–4), but this phosphorylation was abrogated in the Syk-T-infected cells as was the tyrosine phosphorylation of p120 and p140 (Fig. 3B, lanes 5 and 6). In these two experiments there was a disparity in the extent of 32P labeling and tyrosine phosphorylation of p46shc which could be indicative of the phosphorylation of p52shc by protein kinase C which was reported to enhance the interaction of p52shc with cytosolic tyrosine phosphatase (48). The increased association of Shc with Grb2 was still observed in wild type infected cells, was enhanced in Syk-infected cells, and was blocked in Syk-T-infected cells (Fig. 3C).

In contrast to the above results, the small amounts of Grb2 that were constitutively associated with Shc (i.e. unstimulated cells in Fig. 3C, lanes 1, 3, and 5) were unaffected by any of the expression systems. A small increase in the association of Grb2 with Sos was noted in Syk-transfected cells after antigen stimulation (Fig. 3D, lane 4). In general, however, the association of Sos with Grb2 was minimally influenced by antigen stimula-
and the apparent modification of Sos (i.e. retardation in electrophoretic migration) and its dissociation from Shc. It would appear that p120 and p140 are engaged by receptors that possess intrinsic tyrosine kinase activity, such as the EGF receptor (16), or recruit tyrosine kinases, such as FcRII (this paper). The dissociation of Sos from Shc might be due to phosphorylation of Sos by protein kinase C or MAP kinase. As noted earlier, phosphorylation by either enzyme is associated with a retardation in electrophoretic migration of Sos. Phosphorylation by protein kinase C is thought to represent a step in the cycle of activation and inactivation of Sos (18) or, in the case of phosphorylation by MAP kinase, to promote dissociation of the Sos-Grb2 complex from tyrosine-phosphorylated peptides (20). These considerations suggest a variation of the second scenario that was discussed earlier in that tyrosine phosphorylation of Shc promotes formation of a ternary complex with pre-existing Grb2-Sos complexes and subsequent phosphorylation of Sos by protein kinase C promotes dissociation of this complex. Further investigation of these phenomena is currently warranted, but they illustrate features shared by receptors that utilize intrinsic or extrinsic tyrosine kinase activities for initiating stimulatory signals.

REFERENCES

1. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavalli, F., Forni, G., Nicosia, V., Grignani, F., Ponzoni, T., and Pelicci, P. G. (1992) Cell 70, 93–104
2. Skolnik, E. Y., Batzer, A., Li, N., Lee, C. H., Lowenstein, E., Mohammadi, M., Margolis, B., and Schlessinger, J. (1993) Science 260, 1953–1955
3. Skolnik, E. Y., Lee, C. H., Batzer, A., Vicentini, L. M., Zhou, M., Daly, R., Myers, M. J., Jr., Backer, J. M., Ullrich, A., White, M. F., and Schlessinger, J. (1993) EMBO J. 12, 1929–1936
4. Smilt, L., de Vries-Smits, A. M. M., Bos, J. L., and Borst, J. (1994) J. Biol. Chem. 269, 20209–20212
5. Lowy, D. R., and Willumsen, B. M. (1993) Annu. Rev. Biochem. 62, 851–891
6. Lonsdale, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-Sagi, D., and Schlessinger, J. (1992) Cell 70, 431–442
7. Buday, L., and Downward, J. (1993) Cell 73, 611–620
8. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993) Nature 363, 45–51
9. Rozakis-Adcock, M., McGlade, J., Maral, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G., Schlessinger, J., and Ponzoni, T. (1992) Nature 360, 689–692
10. Hu, Z. Q., Yamazaki, T., Cai, Z., Yoshida, T., and Shimamura, T. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 9500–9504
11. Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlessinger, J., and Bar-Sagi, D. (1993) Nature 363, 88–92
12. Quilliam, L. A., Huff, S. Y., Kambo, R. K., Mei, W., Park, W., Brooks, D., and Der, C. J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 8532–8536
13. Aronheim, A., Engelberg, D., Li, N., Al-Alawi, N., Schlessinger, J., and Karin, M. (1994) Cell 78, 949–961
14. Pronk, G. J., de Vries-Smits, A. M., Buday, L., Downward, J., Maassen, J. A., Medema, R. H., and Bos, J. L. (1994) Mol. Biol. Cell 15, 1575–1581
15. de Vries-Smits, A. M., Pronk, G. J., Medema, J. P., Burgering, B. M. T., and Bos, J. L. (1995) Oncogene 10, 919–925
16. Lanfrancone, L., Pelicci, G., Brizzoli, M. F., Aruoa, M. C., Casagrandi, C., Giuli, S., Pegoraro, L., Ponzoni, T., and Pelicci, P. G. (1995) Oncogene 10, 907–917
17. Turner, H., Keif, K., Rivera, J., and Cantriel, D. A. (1995) J. Biol. Chem. 270, 9500–9506
18. Rozakis-Adcock, M., Fernley, R., Wade, J., Ponzoni, T., and Bowtell, D. (1993) Nature 363, 83–85
19. Cherniak, A. D., Klarlund, J. K., and Czech, M. P. (1994) J. Biol. Chem. 269, 4717–4720
20. Buday, L., Warne, P. H., and Downward, J. (1995) Oncogene 11, 1327–1331
21. Williams, M. J., Duronio, R. V., Leslie, K. A., Bowtell, D., and Sardana, J. W. (1994) J. Biol. Chem. 269, 21165–21176
22. Cambier, J. C. (1995) J. Immunol. 155, 3281–3285
23. Ravichandran, K. S., Lee, K. K., Songyang, Z., Cantley, L. C., Lorn, J., and Siraganian, R. P. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 9500–9504
24. Saxton, T. M., van Oostvoo, J., Bowtell, D., Aebbersold, R., and Gold, M. R. (1994) J. Biol. Chem. 153, 623–636
25. Hiraga, N., Scharenberg, A., Yamanaka, H., Bean, M. A., and Kinet, J.-P. (1995) J. Biol. Chem. 270, 10960–10967
26. Eisenman, E., and Bolen, J. B. (1992) Nature 355, 78–80
27. Hunthof, J. E., Gehlen, R. L., Deinlein, G. G., and Oliver, J. M. (1992) Mol. Cell. Biol. 12, 3555–3561
28. Jouvin, M.-H. E., Adamczewski, M., Numerof, R., Letourneur, O., Valle, A., and Siraganian, R. P. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 11246–11250
29. Yamashita, T., Mio, S.-Y., and Metzger, H. (1994) Proc. Natl. Acad. Sci.
32. Scharenberg, A. M., Lin, S., Cuenod, B., Yamamura, H., and Kinet, J. P. (1995) EMBO J. 14, 3385–3394
33. Benhamou, M., Gutkind, J. S., Robbins, K. C., and Siraganian, R. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5327–5330
34. Benhamou, M., and Siraganian, R. P. (1992) Immunol. Today 13, 195–197
35. Benhamou, M., Stephan, V., Robbins, K. C., and Siraganian, R. P. (1992) J. Biol. Chem. 267, 7310–7314
36. Paolini, R., Jouvin, M.-H., and Kinet, J.-P. (1991) Nature 353, 855–858
37. Gulbins, E., Langlet, C., Baier, G., Bonnefoy-Berard, N., Herbert, E., Altman, A., and Coggeshall, K. M. (1994) J. Immunol. 152, 2123–2129
38. Gulbins, E., Coggeshall, K. M., Baier, G., Katzav, S., Burn, P., and Altman, A. (1993) Science 260, 822–825
39. Offermanns, S., Jones, S. V. P., Bombien, E., and Schultz, G. (1994) J. Immunol. 152, 250–261
40. Hirasawa, N., Santini, F., and Beaven, M. A. (1995) J. Immunol. 154, 5391–5402
41. Santini, F., and Beaven, M. A. (1993) J. Biol. Chem. 268, 22716–22722
42. Taniguchi, T., Kobayashi, T., Kondo, J., Takahashi, K., Nakamura, H., Suzuki, J., Nagai, K., Yamada, T., Nakamura, S.-I., and Yamamura, H. (1991) J. Biol. Chem. 266, 15790–15796
43. Graves, J. D., Downward, J., Izquierdo-Pastor, M., Rayter, S., Warne, P. H., and Cantrell, D. A. (1992) J. Immunol. 148, 2417–2422
44. Crowe, A. J., McGlade, J., Pawson, T., and Hayman, M. J. (1994) Oncogene 9, 537–544
45. Donovan, J. A., Wange, R. L., Langdon, W. Y., and Samelson, L. E. (1994) J. Biol. Chem. 269, 22921–22924
46. Fukazawa, T., Reedquist, K. A., Trub, T., Soltow, S., Panchamoorthy, G., Druker, B., Cantley, L., Shokat, S. E., and Band, H. (1995) J. Biol. Chem. 270, 19141–19150
47. Cory, G. O., Lovering, R. C., Hinshelwood, S., MacCarthy-Morrogh, L., Levinsky, R. J., and Kinnon, C. (1995) J. Exp. Med. 182, 613–615
48. Habib, T., Herrera, R., and Decker, S. J. (1994) J. Biol. Chem. 269, 25243–25246
49. Marais, R., Light, Y., Paterson, H. F., and Marshall, C. J. (1995) EMBO J. 14, 3136–3145