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ETHANOL IMPAIRS INSULIN RECEPTOR SUBSTRATE-1 MEDIATED SIGNAL TRANSDUCTION DURING RAT LIVER REGENERATION

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Chronic ethanol exposure inhibits the capacity of the liver to regenerate. Insulin is a potent hepatotrophic factor and it was determined if ethanol interferes with insulin receptor substrate (IRS-1)-protein mediated signal transduction during liver regeneration. Tyrosyl phosphorylation of IRS-1 was strikingly increased prior to the major wave of DNA synthesis in isocaloric pair-fed control rats; a blunted and delayed response was found in ethanol-fed rats. Enzymatic activity of phosphatidylinositol 3-kinase, a Src homology 2 (SH2) domain containing signal transduction molecule was enhanced by the association with tyrosyl phosphorylated IRS-1, whereas in ethanol-fed rats, this activity was greatly diminished and delayed. These results indicate that one potential molecular mechanism whereby ethanol inhibits hepatocyte DNA synthesis is through its action on the IRS-1-mediated signal transduction cascade.

It has been established that chronic ethanol consumption will produce not only direct toxic effects but also will inhibit hepatocyte growth both *in vivo* and *in vitro* (1-5). Little is known, however, regarding the molecular mechanism whereby ethanol impairs the hepatocyte growth process. Insulin has been shown to be a hepatotrophic factor (6-8), and there is considerable evidence to support the requirement of insulin during liver regeneration. Recently, we have cloned the human insulin receptor substrate 1 (IRS-1) gene (9) which is highly homologous to the corresponding gene of the rat (10). The IRS-1 protein has at least 14 potential tyrosyl phosphorylation (TP) sites. Nine of these tyrosine residues are found in YMXM or YXXM (where Y and M refer to tyrosine and methionine, respectively) motifs, which are excellent substrate for tyrosine kinase activity of the insulin receptor β subunit (10) as well as insulin-like growth factor I (IGF-1) receptor β subunit (11). The IRS-1 protein can associate with signal transducing molecules containing Src homology 2 (SH2) domains through these multiple

Abbreviations: IRS-1, insulin receptor substrate 1; IGF-1, insulin-like growth factor 1; SH2, src-homology 2; TP, tyrosyl phosphorylation; PH, 2/3 hepatectomy; PtdIns 3-kinase, phosphatidylinositol 3-kinase; Mab, monoclonal antibody; PCNA, proliferating cell nuclear antigen; PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate.

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tyrosyl phosphorylation (TP) sites (10). Recent work has described that IRS-1 serves as an intracellular substrate for interleukin-4 (IL-4) receptor and participates in IL-4-stimulated mitogenesis in hematopoietic cells (12), indicating the possibility that IRS-1 may be phosphorylated by IL-4 receptor and /or other growth factor(s) receptors on hepatocytes (13).

We have previously demonstrated that IRS-1 protein was highly tyrosyl-phosphorylated prior to the major wave of DNA synthesis during normal rat liver regeneration induced by 2/3 hepatectomy (PH), whereas protein expression of IRS-1 was only slightly increased. In this context, phosphatidylinositol 3-kinase (PtdIns 3-kinase), one of the signal-transducing molecules containing SH2 domains, was highly associated with IRS-1 following TP in vivo (14). These results suggest that TP on IRS-1 protein may play an important role during liver regeneration and transmits the insulin-, IGF-1-, IL-4- induced signals and/or signals induced by other growth factors from the cell surface to intracellular regulator proteins involved in hepatocyte growth. This study was performed to determine if ethanol interferes with IRS-1-mediated signal transduction and therefore impairs liver repair by this mechanism. To do this, we employed an ethanol and isocaloric pair-fed rat model and subjected the animals to PH.

EXPERIMENTAL PROCEDURES

Antibodies Anti-human IRS-1 rabbit polyclonal antibodies were prepared as previously reported (9). Anti-phosphatidylinositol 3-kinase (PtdIns 3-kinase) rabbit polyclonal antibodies were purchased from UBI (Lake Placid, NY). Anti-phosphotyrosine mouse monoclonal antibody (Mab) was kindly provided by Dr. Brian Drucker (Dana-Farber Cancer Institute, Boston, MA). ^{125}I-labeled anti-phosphotyrosine mouse Mab was prepared as previously described (15). Anti proliferating cell nuclear antigen (PCNA) monoclonal antibody (16) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Animal model Adult female CD rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 150-200 g were divided into two groups. One group (n=11) was fed the Lieber-de Carli liquid ethanol diet in which ethanol contributes 36% of the total calories. The second group (n=11) was pair-fed the isocaloric control diet where dextrin-maltose was substituted for ethanol (Bio-Serv. Inc., Frenchtown, NJ). The animals were fed the respective diets for 8 weeks. Repeat experiment was performed with an additional set of ethanol-fed (n=11) and pair-fed control (n=11) groups.

Animal surgery and tissue preparation After 8 weeks on the respective diets, the animals were fasted overnight prior to a 2/3 hepatectomy (PH). Under light ether anesthesia, the median and left lateral lobes were excised according to the method of Higgins and Anderson (17). Partial hepatectomies were performed between 8 and 10 a.m. since the effect of ethanol on liver regeneration is maximal under these conditions (2). After PH, the rats were allowed to recover and consume their respective preoperative diets. Animals were sacrificed and the livers were rapidly excised to prepare tissue homogenates at various times after PH. These protocols were approved by the Subcommittee on Animal Welfare at the Massachusetts General Hospital.

Immunoprecipitation and Western blot analysis Liver tissue homogenates were prepared as previously reported (14).The homogenates (300 μg) were reacted with either anti-IRS-1 rabbit polyclonal antibodies, anti PtdIns 3-kinase rabbit polyclonal antibodies, or anti PCNA Mab at 4°C overnight, and incubated with Protein A sepharose beads (Sigma, St Louis) for additional three hours to precipitate the antibody-antigen complexes. Western blotting analysis was performed as described (14). Antibodies binding to PVDF filters (Millipore, Bedford, MA) were detected with ^{125}I-labeled protein A (DuPont, Boston, MA). ^{125}I-labeled anti-phosphotyrosine mouse Mab was used directly to detect TP of IRS-1. The time-dependent change in protein expression or TP of IRS-1 relative to the initial level (time 0) was determined by densitometric scanning of the autoradiographs.

Assay for phosphatidylinositol-3-kinase (PtdIns 3-kinase) activity PtdIns 3-kinase assay was conducted as previously described (18, 19). Liver tissue homogenates (1mg) was reacted with anti-IRS-1 polyclonal antibodies at 4°C overnight and incubated with Protein A Sepharose beads (Sigma, St Louis) for additional 3 hr to precipitate the antibody-antigen

complexes. The immunoprecipitates were then collected and washed three times with cold phosphate buffered-saline containing 1% Nonidet P40 (Boehringer Mannheim Biochemicals, Indianapolis, IN), three times with 100mM Tris (pH 7.4) with 5mM LiCl, twice with TNE buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5mM EDTA). The above buffers contained 2 mM $NaVO_3$. Immunoprecipitates were then preincubated with 1mg/ml of sonicated phosphatidylinositol (PI) in 20mM HEPES (pH 7.4) 1mM EDTA (pH 7.4) for 20 min at 20°C. After preincubation, the reaction was started by addition of 5 mM ATP, 100mM $MgCl_2$, 2mM Adenosine (final concentrations; 40 mM, 10 mM , 200 mM , respectively) and 40 mCi γ-^{32}P ATP (3,000 Ci/ mmol). Adenosine was added in PtdIns 3-kinase assay to prevent any contaminating phosphatidylinositol 4-kinase activity (18). The reactions were carried out for 20 min at 20 °C and terminated by the addition of 1 N HCl. Products of the reaction were extracted with chloroform/methanol (1:1). The organic phase was twice washed with methanol/HCl (1:1) and spotted onto thin-layer chromatography (TLC) (Silica Gel 60, Merck, Germany) pretreated with 1% potassium oxalate, and developed in chloroform: methanol: water: ammonia (9:7:1.5:0.5, v/v). The radioactive reaction products were visualized by autoradiography at -70°C. Unlabeled phospholipid standards were included and visualized by exposure to iodine vapor. The radioactivity spots corresponding to phosphatidylinositol 3-monophosphate (PIP) were excised and determined by Cerenkov counting.

RESULTS AND DISCUSSION

Tyrosyl phosphorylation of IRS-1 protein The first experiment was designed to examine the influence of chronic ethanol-feeding on TP of IRS-1 protein during liver regeneration, as a possible mechanism for reduced hepatocyte DNA synthesis. TP of IRS-1 was enhanced 12-15 fold over the initial level with two peaks occurring at 2 and 12 hr after PH as shown in pair-fed control rats (Figure 1A). This pattern is identical to a previous finding in animals fed a standard rat chow diet (14) and suggests that the isocaloric liquid diet does not affect TP of IRS-1 during normal rat liver regeneration. In ethanol-fed rats, TP of IRS-1 was reduced 2-3 fold over the initial levels and two small peaks were found at 4-6 and 36 hr after PH (Figure 1B), indicating that chronic ethanol-feeding blunted and delayed TP of the IRS-1 protein. There was no difference in the change of IRS-1 protein expression during liver regeneration between ethanol-fed and pair-fed controls (data not shown).

In order to examine the relationship between TP of IRS-1 and DNA synthesis under these experimental conditions, liver tissue homogenates were subject to immunoprecipitation followed by Western blotting with a Mab directed against the proliferating cell nuclear antigen (PCNA)(16). A previous study has established a good correlation between PCNA expression and nuclear labeling with bromodeoxyuridine after PH in rats (20). Expression of PCNA was highly enhanced with a peak at 24 hr after PH in pair-fed control rats (Figure 1C). In ethanol-fed rats, expression of PCNA was blunted and delayed with the peak shifted to 36 hr (Figure 1D). These observations are in agreement with a previous report using ^{3}H-thymidine corporation as an index of liver regeneration in ethanol and pair-fed control rats (3). Comparison of Figure 1A & B with 1C & D indicate that TP of IRS-1 precedes DNA synthesis both in pair-fed control and ethanol-fed rats, suggesting that TP of IRS-1 may play an important role in the sequential events leading to DNA synthesis. More importantly, a diminished and blunted pattern of TP of IRS-1 correlates well with reduced DNA synthesis in the ethanol-fed rats.

Association of PtdIns 3-kinase with IRS-1 protein and PtdIns 3-kinase activity

The Src homology 2 domains are conserved sequences consisting of approximately 100 amino

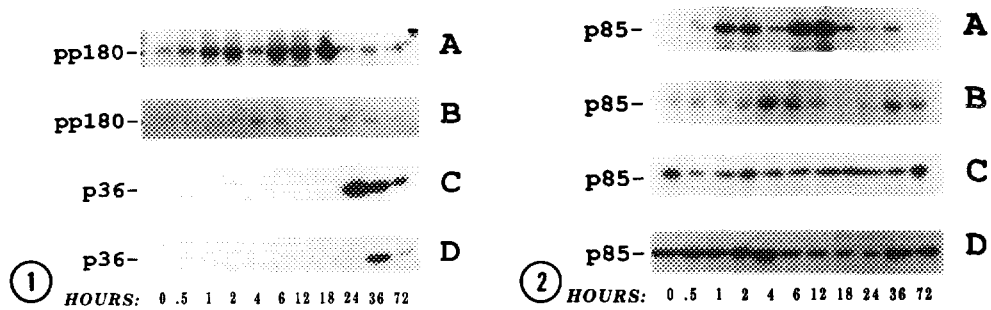


Figure 1. **A - D.** Tyrosyl phosphorylation (TP) of IRS-1 protein during liver regeneration of pair-fed control (A) and ethanol-fed rats (B). Expression of proliferating cell nuclear antigen (PCNA) during liver regeneration in pair-fed controls (C) and ethanol-fed rats (D). PCNA was detected as a protein with a molecular mass of 36 kDa on SDS-PAGE. All results were confirmed with an additional set of pair-fed control (n=11) and ethanol-fed rats (n=11). Numbers below the panels indicate the times after PH.

Figure 2. **A - D.** Association of phosphatidylinositol 3-kinase (PtdIns 3-kinase) with IRS-1 protein during liver regeneration after PH of pair-fed control(A) and ethanol-fed rats (B). Note that association of PtdIns 3-kinase with IRS-1 protein was highly correlated with TP of IRS-1 (compared with Figure 1 A & B). In contrast, the protein levels of PtdIns 3-kinase did not change appreciably during liver regeneration in both pair-fed control (C) and ethanol-fed rats (D). Identical results were obtained with an additional set of pair-fed control rats (n=11) and ethanol-fed rats (n=11). Numbers below the panel indicate the times after PH.

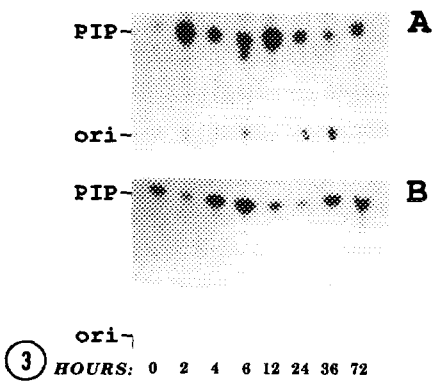
acids and are found among a series of signal transducing molecules (21, 22). Proteins with SH2 domains binds specifically to phosphotyrosine-containing sequences in growth factor receptors and other tyrosyl-phosphorylated proteins implicated in normal signaling as well as in cellular transformation. A previous experiment has demonstrated that PtdIns 3-kinase, one of the signal transducing molecules containing SH2 domain, was highly associated with tyrosyl-phosphorylated IRS-1 and this binding was changing according to the degree of TP of IRS-1 during liver regeneration in normal rats (14). Although the cellular functions of PtdIns 3-kinase are presently unclear, numerous studies on the biological activity of mutant growth factor receptors suggest that PtdIns 3-kinase plays a crucial role in cellular transformation and responses to mitogens (23-26). Thus, the association of PtdIns 3-kinase with IRS-1 may be an important mechanism of insulin action on hepatocyte growth.

The following experiment was designed to reveal if ethanol interferes with association of IRS-1 with PtdIns 3-kinase during liver regeneration *in vivo*. Anti-IRS-1 immunoprecipitates were separated by SDS-PAGE and probed with anti-p85 (PtdIns 3-kinase 85 KDa subunit) antibody. Association of IRS-1 with PtdIns 3-kinase demonstrated a different pattern between pair-fed control rats and ethanol-fed rats; association of IRS-1 with PtdIns 3-kinase was enhanced 6-9 fold over the initial levels with two peaks observed at 2 and 6-12 hr respectively, after PH in controls (Figure 2A). In the ethanol-fed group, this association was not only delayed from 2 to 4 hr, 12 to 36 hr but also the peak was substantially reduced to about 3 fold over the initial level (Figure 2B). Comparison of Figure 1 A & B and Figure 2 A & B demonstrate that association of IRS-1 with PtdIns 3-kinase during liver regeneration occurred in parallel with, and depended on, the state of tyrosyl-phosphorylation of IRS-1 in both pair-fed control and ethanol-fed rats. In contrast, immunoprecipitation followed by Western blot analysis with anti-p85

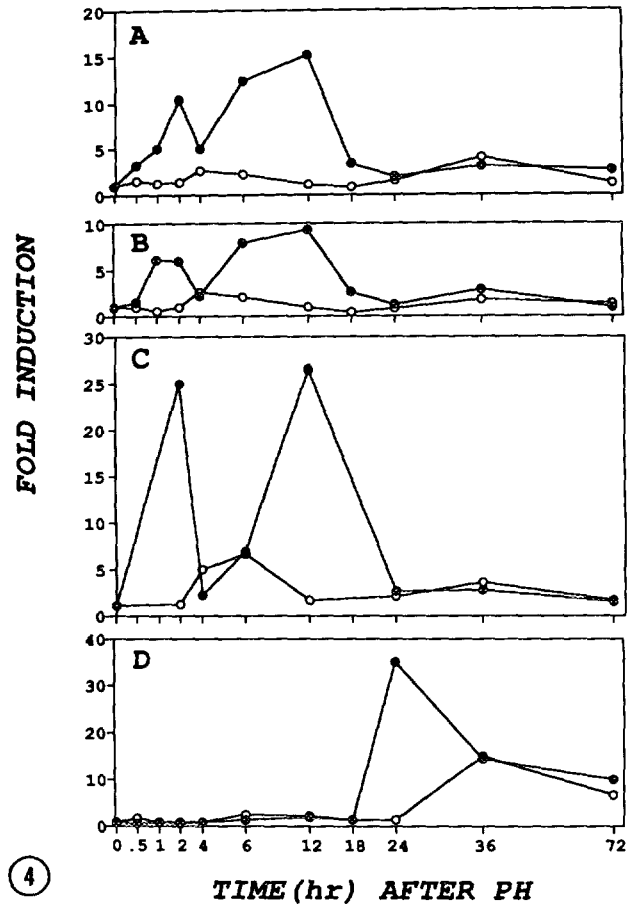
antibodies described that expression of PtdIns 3-kinase protein in regenerating liver was essentially unchanged after PH in both pair-fed control and ethanol-fed rats (0.4-1.1, 0.5-1.1 fold over the initial level, respectively) (Figure 2 C & D).

A newly identified mechanism for PtdIns 3-kinase enzyme activation involves a distinct conformational change in SH2 domain structure induced by binding of tyrosyl-phosphorylated IRS-1 protein, which is transmitted to the catalytic subunit and regulates enzymatic activity by an allosteric mechanism (27), and supports previous findings that association of PtdIns 3-kinase with tyrosyl-phosphorylated IRS-1 is the critical step for activating PtdIns 3-kinase toward the D-3 position of several phosphatidylinositols during insulin stimulation (28, 29). Enzymatic activity was then determined by scraping and counting the phosphatidylinositol 3-monophosphate (PIP) spots from TLC plates. PtdIns 3-kinase activity was strikingly enhanced approximately 20-25 fold over the initial level with two peaks observed at 2 and 12 hr after PH in the pair-fed control group (Figure 3A). In contrast, ethanol-fed rats exhibited a delayed and reduced response of PtdIns 3-kinase activation with two small peaks of 3-5 fold induction over the initial level at 6 and 36 hr respectively (Figure 3B). Consequently, there was a far greater reduction in PtdIns 3-kinase enzymatic activity in the anti-IRS-1 immunoprecipitates than in the protein content of PtdIns 3-kinase as detected in the anti-IRS-1 immunoprecipitates in ethanol-fed animals. The peaks of PtdIns 3-kinase activity were also observed prior to the major wave of DNA synthesis. Furthermore, the higher level of PtdIns 3-kinase activity observed in pair-fed animals was accompanied by higher rates of DNA synthesis compared to ethanol-fed rats (Figure 1 C & D, Figure 3 A & B). It is interesting to note that a mutation in the regulatory region of the insulin receptor has been found to reduce PtdIns 3- kinase activation during insulin stimulation. There was also a parallel decrease in the tyrosyl phosphorylation of IRS-1 protein and a reduced association and activation of PtdIns 3-kinase. Cells bearing this mutation were defective with respect to insulin stimulation of DNA synthesis in vitro (25, 26). Furthermore, emerging evidence suggests that expression of antisense IRS-1 RNA in Chinese hamster ovary cell lines reduces insulin-stimulated TP of IRS-1 and subsequently lowers PtdIns 3-kinase activity; these events are followed by decreased thymidine incorporation into DNA relative to the parental cell line (30). The relationship between activation of PtdIns 3-kinase and cell growth appears to be similar to that observed with other tyrosine kinases such as the PDGF and CSF receptors (23, 24). Taken together, we are led to believe that activation of PtdIns 3-kinase were mediated through tyrosyl-phosphorylated IRS-1 and may be linked to hepatocyte growth in vivo.

A summary of the changes in TP of IRS-1, the association of tyrosyl phosphorylated IRS-1 with PtdIns 3-kinase, subsequent activation of PtdIns 3-kinase enzymatic activity, and expression of PCNA protein during liver regeneration is presented in Figure 4 . These results indicate the sequential cellular events where TP of IRS-1 followed by the association with PtdIns 3-kinase may strikingly enhance PtdIns 3-kinase activity prior to the major wave of DNA synthesis observed during the hepatic repair process. The present findings demonstrate that ethanol diminishes TP of IRS-1 and this event was accompanied by reduced activation of PtdIns 3-kinase, and suggests that ethanol may inhibit hepatocyte DNA synthesis principally by its action on the IRS-1-mediated signal transduction pathway.



(3)



(4)

Figure 3. **A - B.** Phosphatidylinositol 3-kinase (PtdIns 3-kinase) activity measured in anti-IRS-1 immunoprecipitates from pair-fed control (A) or ethanol-fed rats (B) during liver regeneration. The resultant phosphatidylinositol 3-monophosphate (PIP) product was separated by TLC and spots were visualized by autoradiography. The peak activity was associated with tyrosyl phosphorylated IRS-1 (compare with Figure 1). Fold induction of PtdIns 3-kinase activity was measured and expressed as a ratio of experimental values to the initial value (time zero) prior to partial hepatectomy. The results shown in panels A and B were confirmed by an additional set of pair-fed control (n=11) and ethanol-fed rats (n=11). Numbers below the panels indicate the times after PH. Ori and PIP refer to the origin and the phosphatidylinositol monophosphate, respectively.

Figure 4. **A - D.** Summary of the changes in TP of IRS-1 protein (A), the association of tyrosyl phosphorylated IRS-1 protein with PtdIns 3-kinase (B), PtdIns 3-kinase activity in the anti-IRS-1 immunoprecipitates (C), and change in proliferative cell nuclear antigen (PCNA) expression (D) during liver regeneration in pair-fed control (●) and ethanol-fed (○) rats. Densitometric values are expressed as fold induction compared to the initial level before PH(time zero). The values indicated are the mean of two separate experiments. Numbers below the panels indicate the times after PH.

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REFERENCES

1. Lieber, C.S. (1988) N. Engl. J. Med. 319, 1639-1650.
2. Wands, J.R., Carter, E.A., Bucher, N.L.R, Isselbacher, K.J.(1979) Gastroenterology. 77, 528-531.
3. Duguay, L., Coutu, D., Hetu, C., Joly, J.G.(1982) Gut. 23, 8-13.
4. Carter, E.A., Wands , J.R. (1985) Biochem. Biophys. Res. Commun. 128, 767-774.
5. Diehl, A.M., Yang, S.Q., Cote, P., Wand, G.S. (1992) Hepatology. 16, 1212-1219.
6. Starzl, T.E., Francavilla,,A., Halgrimson, C.G., Francavilla, F.R., Poter, K. A., Brown, T.H., Putman,. C.W. (1973) Surg. Gynecol. Obstet .137, 179-199.
7. Bucher, N.L.R., Patel, U., Cohen, S. (1978) Hormonal factors concerned with liver regeneration. In: Hepatotrophic factors, CIBA Foundation Symposium (R. Porter, J. E Whelan, eds.) No.55. pp. 95-110. Amsterdam:Excerpta Medica, North Holland.
8. Bucher, N.L.R., Swaffield, M.N. (1975) Proc. Natl .Acad. Sci. U.S.A. 72, 1157-1160.
9. Nishiyama, M., Wands, J.R.(1992) Biochem. Biophys. Res. Commun. 183, 280-285.
10. Sun, X.J., Rothenberg, P., Kahn, C.R., Backer, J.M,, Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J., White, M.F. (1991) Nature. 352,73-77.
11. Kadowaki, T., Koyasu, S., Nishida, E., Tobe, K., Izumi ,T., Takaku, F., Sakai, H., Yahara, I., Kasuga, M.(1987) J. Biol. Chem.262, 7342-7350.
12. Wang, L.M., Myers, M.G. Jr., Sun, X.J,, Aaronson, S.A., White, M., Pierce, J.H. (1993) Science. 261,1591-1594.
13. Lowenthal, J.W., Castle, B. E., Christiansen, J., Schreurs, J., Rennick, D., Arai, N., Hoy, P., Takebe, Y., and Howard, M. (1988) J. Immunol. 140, 456-464.
14. Sasaki, Y., Zhang, X.F., Nishiyama, M., Avruch, J. Wands, J.R. (1993) J Biol Chem 268, 3805-3808.
15. Harlow, E., and Lane, D. (1988) Antibodies: A laboratory manual, pp. 330-334, Spring Harbor Laboratory, Cold Spring Harbor, NY.
16. Ogata, K., Ogata, Y., Takahashi, Y., Tan, E.M. (1987) J. Immunol.139, 2942-2946.
17. Higgins, G.M,, Anderson, R.M.(1931) Arch. Pathol. 12,186-202.
18. Kaplan, D.R,, Whitman, B., Schaffhausen, D.C., Pallas, D.C., White, M., Cantley, L., Roberts, T.M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 362-364.
19. Escobedo, J.A., Kaplan, D.R., Kavanaugh, W.M., Turck, C.W., Wiliams, L.T. (1991) Mol. Cell .Biol.11, 1125-1132.
20. Wolf, H.K,, Michalopoulos, G.K. (1992) Hepatology. 15, 707-713.
21. Koch, C.A., Anderson, D., Moran, M.F., Ellis, C., Pawson, T. (1991) Science 252, 668-674.
22. Lowenstein, E.J,, Daly, R.J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E,Y., Bar-Sagi, D., Schlessinger, J. (1992) Cell. 70, 431-442.
23. Coughlin, S.R., Escobedo, J.A., Williams, L.T. (1989) Science. 243, 1191-1194.
24. Choudhury, G.G., Wang, L.M., Pierce, J., Harvey, S.A., Sakaguchi, A.Y.(1991) J. Biol. Chem. 266, 8068-8072.
25. Wilden, P.A., Backer, J.M., Kahn, C.R., Cahill, D. A., Schroeder, G.J., White, M.F. (1990) Proc. Natl. Acad. Sci .U.S.A . 87, 3358-3362.
26. Backer, J. M., Schroeder, G. G., Kahn, C. R., Myers, M.G. Jr., Wilden, P.A., Cahill, D. A., White, M.F. (1992) J. Biol. Chem. 267, 1367-1347.
27. Shoelson, S.E., Sivaraja, M., Williams, K.P., Hu, P., Schlessinger, J., Weiss, M.A. (1993) EMBO. J . 12.795-802.
28. Backer, J.M., Myers, M.G.Jr,. Shoelson, S.E., Chin, D.J., Sun, X.J,. Miralpeix, M., Hu, P., Margolis, B., Skolnik, E.Y., Schlessinger, J., White, M.F. (1992) EMBO. J.11, 3469-3479.
29. Myers, M. G. Jr., Backer, J.M., Sun, X.J., Shoelson, S., Hu ,P., Schlessinger, J., Yoakim, M., Schaffhausen, B., White, M.F. (1992) Proc. Natl. Acad. Sci. U.S.A. 89:10350-10354.
30. Waters, S.B., Yamauchi, K., Pessin, J.E. (1993) J. Biol. Chem. 268, 22231-22234.