Immunoprecipitation and Immunoblotting Analysis.

Tissues were dissected and stored frozen at -80°C prior to analysis. Samples (about 0.2g) were suspended in (2ml) lysis buffer containing 50 mM Tris (pH8.0), 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40 (NP40). Protease inhibitors including phenylmethylsulfonyl fluoride (25 mg/ml), leupeptin (0.5 mg/ml), and pepstatin (0.7 mg/ml), were included in the lysis buffer (Boehringer Mannheim Biochemicals, BMB). Tissues were homogenized and sonicated 10s on ice. The tissue extracts were cleared twice by centrifugation at 3000 x rpm for 10-15 min. Protein determinations, immunoprecipitation reactions, and polyacrylamide denaturing gel electrophoresis were performed as described previously (Symonds et al, 1991). I used about 150-300 micrograms of tissue extract per assay (based on OD280). Proteins were transferred to nitrocellulose (Biorad) a minimum of seven hours to overnight at 25 V in 20 mM Tris, 150 mM glycine, 20% methanol (I started first 15' 10 vol, 15' 15 vol, 15' 20 vol and then overnight 25 volt). Do not air dry filters!!!!, rinse them in TBS-NP40 (0.2 M NaCl and 50 mM Tris-Cl, pH7.4, 0.1% NP40) and block with 3% bovine serum albumin (BSA) and 5% dry milk powder in TBS-NP40 for 1h. Filters were incubated for 1-3h at room temperature with monoclonal supernatants or an appropriate primary antibody dilution. Following each antibody incubation, filters were washed 3x with TBS-NP40. The filters were then incubated for 1h at room temperature with (1:3000) dilution of Protein A conjugated-Horseradish peroxidase (Protein A-HRP, Zymed). Following incubation, the filters were rinsed as described above. Specific reactions were detected as recommended using the ECL system (Amersham). The filters were exposed to Kodak film at room temperature generally for 1-5 min.