Identification of pp68 as the Tyrosine-phosphorylated Form of SYNCRIP/NSAP1

A CYTOPLASMIC RNA-BINDING PROTEIN*

Received for publication, March 15, 2002
Published, JBC Papers in Press, May 6, 2002, DOI 10.1074/jbc.M202556200

Richard C. Hresko and Mike Mueckler‡
From the Department of Cell Biology and Physiology, Washington University School of Medicine,
St. Louis, Missouri 63110

Recently we reported that osmotic shock increased the insulin-stimulated tyrosine phosphorylation of a 68-kDa RNA-binding protein in 3T3-L1 adipocytes (Hresko, R. C., and Mueckler, M. (2000) J. Biol. Chem. 275, 18114–18120). In this present study we have identified, by MALDI mass spectrometry, pp68 as the tyrosine-phosphorylated form of synaptotagmin-binding cytoplasmic RNA-interacting protein (SYNCRIP)/NSAP1, a newly discovered cytoplasmic RNA-binding protein. Both SYNCRIP and pp68 were enriched in free polysomes found in low density microsomes isolated from 3T3-L1 adipocytes. In vitro phosphorylation studies revealed that SYNCRIP, once extracted from low density microsomes, can be tyrosine phosphorylated using purified insulin receptor. Binding of RNA to SYNCRIP specifically inhibited its in vitro phosphorylation but had no effect on receptor autophosphorylation or on the ability of the receptor to phosphorylate a model substrate, RCM-lysozyme. These results raise the possibility that regulation of mRNA translation or stability by insulin may involve the phosphorylation of SYNCRIP.

Insulin binding to specific cell surface receptors initiates multiple signaling cascades that lead to a variety of cellular events, including stimulation of glucose and fatty acid uptake, ion and amino acid transport, glycogenesis, lipogenesis, gene transcription, mRNA turnover, protein synthesis and degradation, and DNA synthesis (1). The receptor/hormone interaction results in the autophosphorylation and subsequent activation of the receptor’s intrinsic tyrosine kinase that can then phosphorylate several known cellular substrates, such as the insulin receptor substrate proteins (2), SHC isoforms, Gab-1, Cbl, p60 dok, and adaptor protein containing a PH and SH2 domain (3). Once phosphorylated these proteins serve as docking sites for various Src homology 2 domain-containing proteins that are adaptor proteins themselves, kinases, or phosphatases. The result is the initiation of multiple phosphorylation/dephosphorylation signaling pathways that lead to the various pleiotropic responses.

Recently we found that osmotic shock enhanced by 10-fold the insulin-stimulated tyrosine phosphorylation of a 68-kDa protein in 3T3-L1 adipocytes (4). Further characterization revealed that pp68 was a peripheral protein that resides in a detergent-insoluble fraction of the low-density microsomes (LDM) by binding to RNA. Phosphorylation by insulin was maximal by 1 min and was saturated with 50–100 nM insulin. Activation of the p42/44 and p38 MAP kinase pathways by osmotic shock did not affect pp68 phosphorylation. Based on the failure to immunoprecipitate pp68 with antibodies directed against known 60–70 tyrosine-phosphorylated proteins and on its phosphorylation characteristics, we speculated that pp68 may be a novel cellular target that lies downstream of the insulin receptor whose ability to bind RNA may be crucial in its physiological function.

In the present study we have identified pp68 as the tyrosine-phosphorylated form of SYNCRIP (synaptotagmin-binding cytoplasmic RNA-interacting protein), a recently cloned cytoplasmic 66-kDa RNA-binding protein that has been independently shown to interact with ubiquitous synaptotagmins (5) and be part of a multiprotein complex involved in cytoplasmic RNA turnover (6). We report that SYNCRIP can be phosphorylated in vitro by the insulin receptor tyrosine kinase and that its phosphorylation is regulated by its ability to bind RNA.

EXPERIMENTAL PROCEDURES

Subcellular Fractionation of 3T3-L1 Adipocytes—3T3-L1 fibroblasts were grown to confluence and 48 h later subjected to differentiation as described previously (7). 3T3-L1 adipocytes were used 10–14 days after differentiation. Cells were washed three times with phosphate-buffered saline (PBS) and incubated for at least 2 h to overnight in serum-free Dulbecco’s modified Eagle’s medium (DMEM). Adipocytes were then incubated in DMEM alone or DMEM supplemented with insulin or sorbitol. After the treatment, the cells were washed three times with ice-cold PBS, scraped in 2 ml per 10-cm dish of ice-cold HES (255 mM sucrose, 20 mM HEPES, pH 7.4, and 1 mM EDTA) containing 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and protease inhibitors, and then homogenized by passing the cells 10 times through a Yamato LSC homogenizer at a speed of 1200 rotations/min at 4 °C. Samples were centrifuged at 10000 × g for 5 min to remove nuclei and unbroken cells. Subcellular fractionation was then carried out on the resulting supernatant essentially as described previously (8). The following protease inhibitors were used: 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1 μg/ml pepstatin, 1 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium molybdate, and 1 mM EDTA.

* This work was supported in part by National Institutes of Health Grant DK38495 and the Diabetes Research and Training Center, Washington University Medical School. 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.
‡ To whom correspondence should be addressed: Dept. of Cell Biology and Physiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-4160; Fax: 314-362-7463; E-mail: mike@cellbio.wustl.edu.

1 The abbreviations used are: LDM, low density microsomes; DMEM, Dulbecco’s modified Eagle’s medium; eIF, eukaryotic initiation factor; HDM, high density microsomes; hnRNP, heterogeneous nuclear ribonucleoprotein; PAGE, polyacrylamide gel electrophoresis; PAIP-1, poly(A)-binding protein-interacting protein; PBS, phosphate-buffered saline; PM, post-mitochondrial supernatant; poly(A), polyadenylic acid; SYNCRIP, synaptotagmin-binding cytoplasmic RNA-interacting protein; MALDI, matrix-assisted laser desorption ionization; NSAP1, NS1-associated protein 1.
antipain, 1 μg/ml benzamidine, 5 μg/ml trypsin inhibitor, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride.

**Purification of pp68**—3T3-L1 adipocytes were washed three times with PBS C and then incubated for 16 h at 37°C with the same medium containing 1 mM Na3VO4. Cells were lysed by treatment with DEEM-containing medium supplemented with 600 mM sorbitol for 15 min at 37°C and then 100 mM insulin was added for an additional 5 min. LDM, prepared as described above, were incubated in ice-cold high salt buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 600 mM NaCl, 1 mM sodium vanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and protease inhibitors) for 30 min at 4°C and then centrifuged for 1 h at 200,000 g. The salt concentration of the resulting supernatant was then adjusted to 150 mM NaCl. Tyrosine-phosphorylated proteins from 300 10-cm plates worth high salt-extracted LDM were purified using an anti-phosphotyrosine antibody column as previously described (9). Proteins eluted with 3 mM phenylphosphate were precipitated with 10% trichloroacetic acid (v/v, final concentration) and then purified by SDS-PAGE. pp68 was visualized using Coomassie Blue R-250 stain and then excised.

**MALDI Mass Spectrometry**—Excised pp68 gel pieces were subjected to trypsin digestion with Promega (Madison, WI) sequencing-grade trypsin using a standard procedure developed in the Protein and Nucleic Acid Chemistry Laboratory (PNACL) at Washington University (St. Louis, MO). The resulting mixture of tryptic peptides was analyzed by matrix-assisted laser desorption ionization (MALDI) with time of flight detection mass spectrometry on a Perseptive Biosystems (Foster City, CA) Voyager DE-PRO mass spectrometry work station. Peptide masses were analyzed with Protein Prospector (University of California, San Francisco) using MS-Fit. This analysis gave an unequivocal protein identification based on a clear distinction of the first MOWSE score relative to all other unrelated ranked proteins. The matched peptide masses represented 21% of the amino acid sequence of the identified protein. This identification was later corroborated by nanocapillary electrospray mass spectrometry on a Finnigan (San Jose, CA) LCQ deca ion trap mass spectrometer.

**Preparation of Antibodies**—Full-length SYNCRIP/NSAP1 cDNA was obtained by polymerase chain reaction amplification using a mouse skeletal muscle Marathon Ready cDNA (CLONTech) and then subcloned into Bluescript SK−. Antibodies were generated against the same two regions of SYNCRIP as previously described (5). Rabbits were immunized with either a glutathione S-transferase fusion protein corresponding to the NH2-terminal region of SYNCRIP (amino acids 1–170) or with a peptide (amino acids 140–152) cross-linked to keyhole limpet hemocyanin via an artificial NH2-terminal cysteine residue. The peptide-specific antibody was used for immunoblots because this antibody recognized only the denatured form of the protein. The antibody directed against the NH2-terminal region was used in immunoprecipitation studies.

**Western Blot Analysis**—50 μg of protein were subjected to SDS-PAGE and then transferred to nitrocellulose. Phosphotyrosine-phosphorylated proteins were detected using the monoclonal PY-20 antibody (Transduction Laboratories). SYNCRIP was detected using the peptide-specific antibody described above at a 1:500 dilution. 125I-Labeled secondary antibodies (Amersham Biosciences) were visualized by autoradiography. Radioactive bands were quantified using a PhosphorImager SI analyzer (Amersham Biosciences).

**Immunoprecipitation of SYNCRIP**—0.5 mg of LDM were boiled for 5 min in 1% SDS. Samples were then diluted with 0.5 ml of buffer A (50 mM HEPES, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM sodium vanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and protease inhibitors) containing Triton X-100 such that the final Triton X-100:buffer A was 1:1. After centrifugation, the supernatant was incubated with 10 μl of SYNCRIP antibody. 50 μl of protein A-Sepharose was then added for 2 h at 4°C to the reactions. After washing pellets four times with ice-cold buffer A containing 1% Triton X-100 and twice with buffer A without detergent, proteins were eluted with SDS-PAGE sample buffer. The immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blot using PY-20 anti-phosphotyrosine antibodies as described above.

**Isolation of Free Polysomes**—Free polysomes were prepared using a modified procedure of Cardelli and Pitot (10). 5–10-cm dishes of 3T3-L1 adipocytes were washed three times with PBS and incubated for at least 2 h to overnight in serum-free DMEM. Cells were then incubated for 30 min in DMEM alone, or in DMEM supplemented with 100 mM insulin, 600 mM sorbitol, or a combination of insulin and sorbitol. Adipocytes were washed three times with ice-cold PBS and then three times with ice-cold 0.44 M STKM (sucrose molarity as designated, 50 mm Tris-HCl, 25 mM KCl, and 5 mM MgCl2, 1 mM sodium vanadate, pH 7.4) containing protease inhibitors. After removal of the buffer, cells were scraped and then disrupted using a ball homogenizer. A postmitochondrial supernatant (PMS) was prepared by centrifugation at 10,000 × g for 10 min at 4°C in a TLA-100.3 Beckman rotor. 2 μl STKM was added to the PMS to a final sucrose concentration of 1.35 M. A discontinuous sucrose gradient was prepared in a 4 ml sealed polyallomer tube. 1.6 ml of PMS (1.35 M) was layered over 1.6 ml of 2 M STKM. 0.44 M STKM was then layered on top of the 1.35 M PMS. Samples were centrifuged at 266,000 × g for 4.5 h at 4°C in a NVT 90 Beckman rotor. Free polysomes were found as a clear pellet near the bottom of the tube.

**In Vitro Phosphorylation of SYNCRIP Using Purified Insulin Receptor**—Wheat germ agglutinin (WGA)-purified insulin receptor was prepared from 3T3-L1 adipocytes as previously described (11). In some experiments 0.5 μg of cytoplasmic domain of the β-subunit of the insulin receptor (Calbiochem) were used in the in vitro reactions. SYNCRIP was immunoprecipitated from 500 μl of DMEM using 15 μl of the glutathione S-transferase fusion SYNCRIP antibody. 1 μM insulin was added or not for 20 min to an aliquot of wheat germ agglutinin-purified insulin receptor (10 μg of total protein in 0.1% Triton X-100). Receptors were autoprophorylated for 5 min at room temperature in 50 mM HEPES (pH 6.9), 100 mM NaCl, 0.1% Triton X-100, 5 mM manganese acetate, and 1 mM ATP. Detergent was omitted in the reactions that contained the cytoplasmic domain receptor. The activated receptor was then added to the immunoprecipitated SYNCRIP, incubated for 15 min, and then quenched with the addition of EDTA (20 mM final) and SDS-PAGE sample buffer. Tyrosine-phosphorylated proteins were analyzed by Western blot analysis. In some reactions, RCM-lysozyme (Sigma) was used as a substrate.

**RESULTS**

**Identification of pp68**—pp68 is a 68-kDa RNA-binding protein that resides in the LDM of 3T3-L1 adipocytes whose insulin-stimulated phosphorylation is dramatically increased with hypertonic stress (4). Based on the elimination of known tyrosine-phosphorylated proteins and on the time and concentration dependence of phosphorylation, we previously concluded that pp68 may be a novel substrate of the insulin receptor tyrosine kinase whose ability to bind RNA may dictate its physiological function. To identify this protein, pp68 was purified to homogeneity from 300 10-cm dishes of 3T3-L1 adipocytes using phosphotyrosine affinity chromatography and SDS-PAGE as described under “Experimental Procedures.” Purified pp68 was excised from Coomassie Blue-stained polyacrylamide gels, trypsinized, and then analyzed by MALDI mass spectrometry. pp68 was unequivocally identified as being one of three highly homologous RNA-binding proteins, SYNCRIP, NSAP1, or heterogeneous nuclear ribonucleoprotein R (hnRNP R). The peptide sequences of SYNCRIP, which represent 21% of the protein, identified by MALDI based on matched peptide masses are shown in Table I. SYNCRIP is a recently cloned cytoplasmic 66-kDa mouse RNA-binding protein that was reported to interact with ubiquitous synaptotagmins (5). NSAP1 was identified from a two-hybrid screen as a cellular interactor of NS1, the major nonstructural parovirus protein (12). 99% amino acid identity between NSAP1 and SYNCRIP indicates that NSAP1 is the human homologue of SYNCRIP. hnRNP R, an 82-kDa protein localized in the nucleoplasm, was identified using autoimmune antibodies (13). SYNCRIP is 81.2% identical to hnRNP R but lacks ~70 carboxyl-terminal amino acids that contain a nuclear localization motif (5). Based on similarity in mouse and human and the cytoplasmic localization of pp68, we conclude that pp68 is the mouse protein homolog of SYNCRIP/NSAP1 and not that of hnRNP R. Both SYNCRIP/NSAP1 and hnRNP R contain three sets of RNA recognition motifs with well conserved RNP-1 and RNP-2 submotifs (5). In addition, both possess COOH termini enriched in arginine and glycine residues that have seven Arg-Gly-Gly (RGG) repeats. RGG boxes, another type of RNA-binding motif (14, 15), are also involved in protein-protein interactions (15). Interspersed
between several of the RGG repeats were regions enriched in tyrosine and acidic residues that contain three copies of the tetrapeptide YYGY. These tyrosine-rich regions contain several consensus sites for tyrosine phosphorylation in which acidic residues are found on the NH$_2$-terminal side of the tyrosine (2, 11).

To rule out the possibility that SYNCRIP was an abundant contaminating protein in the pp68 preparation, we immunoprecipitated SYNCRIP from LDM that were first boiled in SDS to disrupt intermolecular interactions, and then analyzed SYNCRIP for phosphotyrosine content by Western blot analysis. 3T3-L1 adipocytes were treated with sorbitol and insulin as described in the legend to Fig. 1. A phosphotyrosine Western blot of immunoprecipitated SYNCRIP from Western blot analysis. Because the different treatments gave exactly the same phosphotyrosine pattern observed for pp68 in total LDM. These results indicate that pp68 is the tyrosine-phosphorylated form of SYNCRIP. The same amount of SYNCRIP was immunoprecipitated under all conditions (Fig. 1C), indicating that the amount of SYNCRIP in the LDM fraction did not change with either sorbitol or insulin treatment.

**Subcellular Localization of SYNCRIP and pp68**—Previously we demonstrated that pp68 resides in a detergent-insoluble fraction of LDM by binding to RNA (4). To determine the extraction properties of non-tyrosine-phosphorylated SYNCRIP, LDM from basal cells were treated with 1% Triton X-100 or RNase A. The soluble and insoluble fractions were isolated after centrifugation for 1 h at 200,000 × g. Western blots of the different fractions using SYNCRIP antibodies (Fig. 2) revealed that like pp68, nearly all of the non-phosphorylated SYNCRIP was found in the detergent-insoluble fraction of the LDM and almost all could be released from the LDM with RNase A treatment.

Next, we fractionated the adipocytes to determine which intracellular compartments were enriched in SYNCRIP. 3T3-L1 adipocytes were treated for 30 min with DMEM alone, or in DMEM supplemented with 100 nM insulin, 600 mM sorbitol, or a combination of insulin and sorbitol and then were fractionated by differential centrifugation as described under “Experimental Procedures.” Western blot analyses of the different fractions were carried out using both SYNCRIP and phosphotyrosine antibodies. Because the different treatments had no effect on the subcellular distribution of SYNCRIP and pp68, only the fractionation results using sorbitol- and insulin-treated cells are shown (Fig. 3). SYNCRIP was found highly enriched in LDM and to a lesser extent in high density microsomes (HDM) and the nucleus. The ~80-kDa protein recognized by the SYNCRIP antibodies in the nuclear fraction is most likely hnRNP R. The phosphotyrosine blot showed that pp68 was highly enriched in LDM and somewhat in HDM.

Based on its subcellular localization and on its affinity for RNA, we tested whether SYNCRIP was associated with polysomes. Polysomes are mRNA molecules associated with multiple ribosomes actively synthesizing nascent proteins. These RNA-protein complexes are membrane-bound in HDM (16) and free in solution in LDM. Free polysomes were isolated from basal, insulin, sorbitol, or sorbitol and insulin-treated adipocytes using high speed centrifugation through a 2 M sucrose cushion as described under “Experimental Procedures” and then analyzed for SYNCRIP protein by Western blot analysis.

---

**Table I**

SYNCRIP/NSAP1 peptide sequences identified by MALDI mass spectrometry from trypsinized pp68

| Peptide | Measured mass | Computed mass | Residues SYNCRIP (NSAP1) peptide sequence |
|---------|---------------|---------------|-------------------------------------------|
| 1       | 633.3801      | 633.4088      | 339-343 (KVLFLVRN(N))                     |
| 2       | 927.4706      | 927.5052      | 185-192 (KAQPIWDLR(L))                    |
| 3       | 941.4406      | 941.3708      | 357-379 (KAMEEMNGKR(D))                   |
| 4       | 1059.4659     | 1059.5383     | 242-249 (KLYNNHERR(S))                    |
| 5       | 1311.5662     | 1311.6545     | 131-142 (RFGTYLTVTTGQR(K))                |
| 6       | 1553.6543     | 1553.7753     | 370-381 (KLKDYAFIHFDR(D))                 |
| 7       | 1708.6968     | 1708.8546     | 67-81 (K(EFNEDGALAV:LQ:QFR(D))            |
| 8       | 1942.7821     | 1942.9762     | 266-282 (K(FRAGTLTVLHQP:RDRK)             |
| 9       | 2014.8465     | 2015.0086     | 43-60 (K(LDEHYAQLVIAHSDL(DER(A)                  |
| 10      | 2557.9058     | 2557.0542     | 425-444 (KNQMYDDYYYYGPHMPPTR(G)            |

---

R. C. Hresko and M. Mueckler, unpublished observation.

---

**Fig. 1. pp68 is the phosphorylated form of SYNCRIP/NSAP1.**

3T3-L1 adipocytes were incubated in DMEM alone (B), DMEM supplemented with 100 nM insulin for 5 or 30 min (I(5) and I(30), respectively), in DMEM containing 600 mM sorbitol for 20 min (S(20)), in DMEM containing 600 mM sorbitol for 15 min followed by an additional 5 min with 100 nM insulin (S(20)/I(5)), or in DMEM containing sorbitol and insulin for 30 min (S(I30)). LDM were then prepared from each of the treated cells. A, LDM were separated by SDS-PAGE (50 μg of protein) and then immunoblotted using an anti-phosphotyrosine antibody. B, 500 μg of LDM from each of the treated cells were boiled in 1% SDS, then diluted in 1% Triton X-100 and then immunoprecipitated with a SYNCRIP-specific antibody. Western blot analysis on the immunoprecipitated was carried out using an anti-phosphotyrosine antibody. C, immunoblot shown in panel B was reprobed with an antibody directed against SYNCRIP.
osmotic shock ribosomes can still be pelleted through a 2 M sucrose cushion, whereas proteins that associate with mRNA like SYNCRIP and PAIP-1 cannot. To determine whether pp68 was also found in free polysomes, SYNCRIP was immunoprecipitated from free polysomes isolated from each of the treated cells and then analyzed for phosphotyrosine content by Western blot analysis. The relative amounts of SYNCRIP protein immunoprecipitated in each of the reactions were determined by reprobing the phosphotyrosine (P-TYR) blot using a SYNCRIP-specific antibody. AB represents a mock immunoprecipitation reaction containing the SYNCRIP antibody without the polysome sample.

**Fig. 4. SYNCRIP is highly enriched in free polysomes.** 3T3-L1 adipocytes were incubated for 30 min in DMEM (B), DMEM supplemented with 100 mM insulin (S), DMEM containing 600 mM sorbitol (S/I), or DMEM containing both 600 mM sorbitol and 100 mM insulin (S/I) and then homogenized. Free polysomes (POLY) were isolated from a PMS by centrifugation through a 2 M sucrose cushion as described under “Experimental Procedures.” A, 20 μg of whole cell lysate (WC), PMS, and free polysomes (POLY) were analyzed by Western blot analysis using SYNCRIP, PAIP-1, and S6 antibodies. B, 200 μg of free polysomes isolated from each of the treated cells were boiled in SDS for 5 min and then diluted with 1% Triton X-100. Samples were then immunoprecipitated using a SYNCRIP-specific antibody and analyzed for phosphotyrosine content by Western blot analysis. The relative amounts of SYNCRIP protein immunoprecipitated in each of the reactions were determined by reprobing the phosphotyrosine (P-TYR) blot using a SYNCRIP-specific antibody. AB represents a mock immunoprecipitation reaction containing the SYNCRIP antibody without the polysome sample.

---

**Fig. 2. SYNCRIP associates with a Triton X-100 (TX-100)-insoluble fraction of the LDM by binding to RNA.** LDM were prepared from basal 3T3-L1 adipocytes. 75 μg of LDM were left either untreated, were solubilized in 1% Triton X-100 for 30 min at 4 °C, or were incubated in 0.4 mg/ml RNase A for 2 h on ice. Soluble (S) and insoluble (P) materials were isolated after centrifugation for 1 h at 200,000 g. The distributions of SYNCRIP for each of the treatments was determined by Western blot analysis using a SYNCRIP-specific antibody. The distribution of pp68 isolated from sorbitol and insulin-treated cells is shown for comparison. P-TYR, phosphotyrosine.

**Fig. 3. SYNCRIP is mostly found in LDM and to a lesser extent HDL and nuclei.** 3T3-L1 adipocytes were treated for 30 min with 600 mM sorbitol and 100 mM insulin. Cells were then fractionated by differential centrifugation as described under “Experimental Procedures.” 50 μg of each of the different subcellular fractions, whole cell lysate (WC), nuclei (NUC), HDL, mitochondria (MITO), plasma membrane (PM), LDM, and cytosol (CYT), were analyzed by Western blot analysis for SYNCRIP and phosphotyrosine (P-TYR) content. Arrows represent the migrations of SYNCRIP and pp68, respectively.

(Fig. 4A). SYNCRIP was enriched about 7.5-fold in free polysomes isolated from basal or insulin-treated cells compared with that in total lysates. There was approximately half the amount of SYNCRIP found in free polysomes from sorbitol ± insulin-treated cells. This is consistent with the fact that hypertonic stress is known to dissociate ribosomes from free polysomes (17, 18). SYNCRIP:NSAP1 has been reported to be part of a multiprotein complex that includes both poly(A)-binding protein and poly(A)-binding protein-interacting protein (PAIP-1) (6). Like SYNCRIP, PAIP-1 was enriched in free polysomes (5.1-fold) isolated from basal or insulin-treated cells (Fig. 4A). Sorbitol treatment dramatically reduced the amount of PAIP-1 in the free polysomes. Our attempts to co-immunoprecipitate PAIP-1 and SYNCRIP in our system, however, were unsuccessful (data not shown). The ribosomal protein S6 was enriched 58-fold in free polysomes, illustrating the extent of purity of our free polysome preparation. The quantity of S6 protein in free polysomes did not change with sorbitol treatment, indicating that when free polysomes are dissociated by
were centrifuged for 1 h at 200,000 \( \times g \) to remove insoluble material. SYNCRIP was immunoprecipitated from 1 mg of cytosol, 500 \( \mu \)g of intact LDM, or once it was removed from 500 \( \mu \)g of LDM using RNase or high salt treatment as described above. Immunoprecipitated SYNCRIP was phosphorylated using the cytoplasmic domain of the \( \beta \)-subunit of the insulin receptor as described under “Experimental Procedures.” A, reactions were analyzed using phosphotyrosine Western blot analysis. B, blot shown in panel A was reprobed using a SYNCRIP-specific antibody.

was significantly less than that purified from LDM (Fig. 5B).

Based on the result that extracting SYNCRIP from the LDM with RNase treatment increased its phosphorylation by the insulin receptor, we tested whether the addition of RNA to the in vitro phosphorylation reaction could specifically inhibit SYNCRIP phosphorylation. LDM was first treated with 600 mM NaCl to extract SYNCRIP from LDM. After the insoluble material was removed by centrifugation and the salt concentration diluted to 150 mM NaCl, SYNCRIP was immunoprecipitated and used as a substrate in the in vitro phosphorylation reaction. The addition of poly(A) RNA completely abrogated insulin-stimulated tyrosine phosphorylation of SYNCRIP (Fig. 6). In contrast, autophosphorylation of the insulin receptor and its ability to phosphorylate a model substrate, RCM-lysozyme, in an insulin-independent manner was not significantly affected by poly(A) RNA.

**DISCUSSION**

In the present study we have identified pp68 as being the tyrosine-phosphorylated form of SYNCRIP/NSAP1 by MALDI mass spectrometry. SYNCRIP is a cytoplasmic RNA-binding protein that has been reported to interact with ubiquitous synaptotagmins (5). Although the significance of this association is not known, the authors (5) of this particular study proposed that non-neuronal synaptotagmins could be involved in organelle-based mRNA transport through its interaction with SYNCRIP. Our results, however, showed that SYNCRIP and pp68 were predominantly localized to LDM through their RNA binding (Fig. 2) and not through their interaction with an integral membrane protein such as synaptotagmin. Therefore, we decided not to focus our attention on the association between SYNCRIP and synaptotagmins in our system.

Further characterization of the localization of SYNCRIP revealed that both SYNCRIP and its phosphorylated form, pp68, were found enriched in free polysomes (Fig. 4). mRNAs are translated in circular form. Recent evidence indicates that the association between the cap structure (\( m^7 \)GpppX, where X represents any nucleotide) of the 5′ terminus and the 3′ poly(A) tail is required for efficient translation initiation (19, 20). The cap, which directs the translation machinery to the 5′ end, binds to the eIF4F complex. eIF4F is a three-component complex comprising the cap-binding protein eIF4A, the RNA helicase eIF4A, and eIF4G, a large scaffolding protein which interacts with ribosome-associated eIF3, eIF4E, and eIF4A. The actual linkage between the two termini in mammalian cells is thought to be through PAIP-1, a protein that simultaneously acts with ribosome-associated eIF3, eIF4E, and eIF4A. The complex comprising the cap-binding protein eIF4E, the RNA helicase eIF4A, and eIF4G, a large scaffolding protein which interacts with ribosome-associated eIF3, eIF4E, and eIF4A. The actual linkage between the two termini in mammalian cells is thought to be through PAIP-1, a protein that simultaneously interacts with poly(A)-binding protein and eIF4A (21). Loss of the 3′ poly(A) tail greatly reduces the rate of translation (20, 22). In addition to promoting efficient translation initiation, the interaction between the two termini is important in maintaining the integrity of an mRNA (19). Proteins that bind to the poly(A) tail and the 5′ cap are thought to protect mRNA from deadenylation and decapping (23), events which precede the 5′ to 3′-exonucleolytic degradation of the mRNAs (24). Short half-life mRNAs contain adenylate, uridylate-rich (AU-rich) instability elements (25) in their 3′-untranslated regions, as well as regions of instability within the protein-coding region (23) that regulate the removal of the poly(A) tail. SYNCRIP/NSAP1 along with Unr, a purine-rich RNA-binding protein, poly(A)-binding protein, PAIP-1, and hnRNPD, an AU-rich element-binding protein, have been reported to form a multiprotein complex that binds to the major protein-coding region determinant of instability of c-fos (6). It has been postulated that formation of this complex would bridge the poly(A) tail and the major protein-coding region determinant of instability and perhaps stabilize the mRNA and that transit of the ribosome might disrupt or reorganize the complex and lead to the deadenylation and subsequent degradation of the mRNA. Contrary to this published report (6), we were unable to coimmunoprecipitate SYNCRIP from an RNase A-treated lysate using an antibody directed against PAIP-1 even though we could demonstrate efficient PAIP-1 immunoprecipitation (data not shown). We did, however co-localize SYNCRIP and PAIP-1 to polysomes, suggesting that these proteins may associate with the same mRNA but in an indirect manner at least in our system.

In our initial study (4), we proposed that p68 (SYNCRIP) could be a direct substrate of the insulin receptor tyrosine phosphorylation system. However, the addition of poly(A) RNA in the in vitro phosphorylation reaction completely abrogated SYNCRIP phosphorylation (Fig. 6). Therefore, we decided not to focus our attention on the association between SYNCRIP and synaptotagmins in our system.
kinase. In vitro phosphorylation studies revealed that SYNCRIP, once extracted from the LDM, could be directly phosphorylated using purified insulin receptor, whereas SYNCRIP isolated from cytosol was poorly phosphorylated (Fig. 5). Binding of RNA specifically inhibited insulin-stimulated SYNCRIP phosphorylation in vitro but had no effect on receptor autophosphorylation or on the ability of the receptor to phosphorylate RCM-lysozyme (Fig. 6). Previously we reported that both osmotic shock and arsenite treatment greatly enhanced the tyrosine phosphorylation of SYNCRIP in 3T3-L1 adipocytes (4). It is also known that osmotic stress and arsenite treatment rapidly blocks protein synthesis at the initiation step leading to the formation of 80S monoribosomes that are free of mRNA (17, 18). The dramatic reduction by sorbitol treatment in the amount of SYNCRIP and PAIP-1 but not ribosomal S6 protein that could be pelleted through a 2 M sucrose cushion (Fig. 4) confirmed this observation. Our working hypothesis based on the phosphorylation results is the following. SYNCRIP can be phosphorylated by the insulin receptor when it is not bound to RNA. Osmotic shock and arsenite treatment leads to the dissociation of free polysomes and increases the amount of mRNA-free SYNCRIP in the cell. Our failure to observe a difference in tyrosine phosphorylation between SYNCRIP samples isolated from cytosol prepared from either basal or sorbitol-treated cells most likely indicates that during the homogenization/subcellular fractionation procedure using sucrose-containing buffers, free SYNCRIP can reassociate with RNA.

SYNCRIP is not the first reported hnRNP protein phosphorylated in response to extracellular signals. hnRNP A1 is predominantly localized in the nucleus but can shuttle between the nucleus and cytoplasm (26). Osmotic shock and UVC irradiation induces the serine phosphorylation of hnRNP A1 via the p38 MAP kinase pathway and causes a redistribution of the protein from the nucleus and cytoplasm (26). Osmotic shock and arsenite treatment greatly enhanced the tyrosine phosphorylation of SYNCRIP in 3T3-L1 adipocytes (4). It is not known due in part to the lack of a clear functional role for another type of RNA-binding motif that is also involved in protein-protein interactions (15). Interspersed between the RGG boxes are several consensus-type sites for tyrosine phosphorylation. RNA-binding proteins not only bind RNA, they can interact with other RNA-binding proteins to form multi-protein complexes as has been previously reported for SYNCRIP. Phosphorylation, therefore, could either affect the affinity of SYNCRIP to bind a specific sequence of RNA or modify its ability to associate with other binding proteins.

Insulin is known to regulate both mRNA translation and stability (35). Protein synthesis is globally induced severalfold with insulin, whereas some individual mRNAs are regulated more dramatically. Insulin can destabilize mRNAs encoding some proteins including: PEPCK, GLUT4, and glycogen synthase, whereas stabilize other messages such as for GLUT1, glyceraldehyde-3-phosphate dehydrogenase, malic enzyme, and phosphorylase (35). Similar to the recent findings for hnRNP K, it is possible that the phosphorylation of SYNCRIP could be involved in the regulation of mRNA translation or mRNA turnover by insulin.

Acknowledgments—We thank Dr. N. Sonenberg for kindly providing PAIP-1 and Dr. Gregory Grant for many useful discussions on MALDI mass spectrometry and the Protein and Nucleic Acid Chemistry Laboratory at Washington University for carrying out the MALDI analysis.

REFERENCES

1. Saltiel, A. R., and Kahn, C. R. (2001) Nature 414, 799–806
2. White, M. F. (1998) Mol. Cell. Biochem. 182, 3–11
3. Pessin, J. E., and Saltiel, A. R. (2000) J. Clin. Invest. 106, 165–169
4. Hresko, R. C., and Mueckler, M. (2000) J. Biol. Chem. 275, 18114–18120
5. Mizutani, A., Fukuda, M., Iwata, K., Shiraishi, Y., and Mikoshiba, K. (2000) J. Biol. Chem. 275, 9822–9831
6. Grosset, C., Chen, C.-Y. A., Xu, N., Sonenberg, N., Jacquemin-Sablon, H., and Shyu, A.-B. (2000) Cell 101, 49–59
7. Tordjman, R. M., Leingang, K. A., James, D. E., and Mueckler, M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7761–7765
8. Pfeiffer, R. C., Hess, L. J., and James, D. E. (1991) Am. J. Physiol. 260, C570–C580
9. Keller, S. R., Kitagawa, K., Aebbersold, R., Linder, G. E., and Garner, C. W. (1991) J. Biol. Chem. 266, 12817–12820
10. Cardelli, J., Long, B., and Pitot, H. C. (1976) J. Cell Biol. 70, 47–58
11. Flores-Riveros, J. R., Sibley, E., Kastelic, T., and Lane, M. D. (1989) J. Biol. Chem. 264, 21557–21572
12. Harris, C. E., Boden, R. A., and Astell, C. R. (1999) J. Biol. Chem. 274, 72–80
13. Hassfeld, W., Chen, E. K. L., Mathison, D. A., Portman, D., Dreyfuss, G., Steiner, G., and Tan, E. M. (1998) Nucleic Acids Res. 26, 439–445
14. Burd, C. G., and Dreyfuss, G. (1994) Science 265, 615–621
15. Dreyfuss, G., and Steiner, G. (1993) Annu. Rev. Biochem. 62, 289–321
16. Clark, S. F., Martin, S., Carozzi, A. J., Hill, M. M., and James, D. E. (1998) J. Cell Biol. 140, 1211–1225
17. Wengler, G., and Wengler, G. (1972) Eur. J. Biochem. 27, 162–173
18. Saborio, J. L., Pong, S.-S., and Koch, G. (1987) J. Mol. Biol. 185, 195–211
19. Gallie, D. R. (1998) Gene (Amst.) 216, 1–11
20. Gallie, D. R. (1991) Genes Dev. 5, 2108–2116
21. Clark, A. W. B., Haghhiat, A., Yu, A. T. K., and Sonenberg, N. (1998) Nature 392, 520–523
22. Munroe, D., and Jacobson, A. (1990) Mol. Cell. Biol. 10, 3441–3455
23. Ross, J. (1995) Microbiol. Rev. 59, 423–450
24. Mitchell, P., and Tollervey, D. (2000) Curr. Opin. Genet. Dev. 10, 189–198
25. Chen, C.-Y., and Shyu, A.-B. (1995) Trends Biochem. Sci. 20, 465–470
26. Palofroma, S., and Dreyfuss, G. (1992) Nature 355, 730–732
27. van der Houven van Oordt, W., Diaz-Meco, M. T., Lozano, J., Krainer, A. R., Moscat, J., and Caseras, J. F. (2000) J. Cell Biol. 140, 307–316
28. Bomsztyk, K., Van Steuven, I., Suzuki, H., Denisenko, O., and Ostrowski, J. (1997) FEBS Lett. 403, 113–115
29. Ostrowski, J., Schullery, D. S., Denisenko, O. N., Higaki, Y., Watts, J., Aebbersold, R., Stempka, L., Geschwendi, M., and Bomsztyk, K. (2000) J. Biol. Chem. 275, 3619–3628
30. Shullery, D. S., Ostrowski, J., Denisenko, O. N., Stempka, L., Shyrveva, M., Suzuki, H., Geschwendi, M., and Bomsztyk, K. (1999) Biochem. J. 344, 15101–15109
31. Habelhah, H., Shah, K., Huang, L., Burlingame, A., Shokat, K., and Ronai, Z. (2001) J. Biol. Chem. 276, 18090–18098
32. Van Steuven, I., Ostrowski, J., and Bomsztyk, K. (1998) Biochemistry 37, 5644–5650
33. Ostrowski, J., Kawata, Y., Schullery, D. S., Denisenko, O. N., Higaki, Y., Abrash, C. K., and Bomsztyk, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9044–9049
34. Shyu, A.-B., and Wilkinson, M. F. (2000) Cell 102, 135–138
35. O’Brien, R. M., and Granner, D. K. (1996) Physiol. Rev. 76, 1110–1145
Identification of pp68 as the Tyrosine-phosphorylated Form of SYNCRI"P/NSAP1: A CYTOPLASMIC RNA-BINDING PROTEIN
Richard C. Hresko and Mike Mueckler

J. Biol. Chem. 2002, 277:25233-25238.
doi: 10.1074/jbc.M202556200 originally published online May 6, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M202556200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 17 of which can be accessed free at http://www.jbc.org/content/277/28/25233.full.html#ref-list-1