NSP1 Defines a Novel Family of Adaptor Proteins Linking Integrin and Tyrosine Kinase Receptors to the c-Jun N-terminal Kinase/Stress-activated Protein Kinase Signaling Pathway

(Received for publication, December 28, 1998, and in revised form, January 29, 1999)

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As part of a program to further understand the mechanism by which extracellular signals are coordinated and cell-specific outcomes are generated, we have cloned a novel class of related adaptor molecules (NSP1, NSP2, and NSP3) and have characterized in more detail one of the members, NSP1. NSP1 has an Shc-related SH2 domain and a putative proline/serine-rich SH3 interaction domain. Treatment of cells with epidermal growth factor or insulin leads to NSP1 phosphorylation and increased association with a hypophosphorylated adaptor protein, p130Cas. In contrast, cell contact with fibronectin results in Cas phosphorylation and a transient dissociation of NSP1 from p130Cas. Increased expression of NSP1 in 293 cells induces activation of JNK1, but not of ERK2. Consistent with this observation, NSP1 increases the activity of an AP-1-containing promoter. Thus, we have described a novel family of adaptor proteins, one of which may be involved in the process by which receptor tyrosine kinase and integrin receptors control the c-Jun N-terminal kinase/stress-activated protein kinase pathway.

Cell contact with growth factors and extracellular matrix activates one or more of the MAPK pathways, and these responses appear to be critical for the appropriate regulation of cell division and differentiation (1, 2). Although many of the molecules that participate in these processes have been defined, there are still significant gaps in our understanding of how multiple signals are integrated into an appropriate outcome.

The MAPK kinase family of proteins (ERK, JNK, and p38) can phosphorylate and activate one or more transcription factors, Cas may well assemble a number of proteins such as FAK and the adaptor molecule Cas (Crk-associated substrate) (20–22). FAK is primarily responsible for phosphorylating a number of proteins involved in cytoskeletal assembly (23, 24), whereas c-Src (or Src-like kinases) can phosphorylate Cas (25) and can activate the MAPK pathway (26, 27). Cas, originally identified as a hyperphosphorylated protein following induced expression of the viral oncogene Crk (v-Crk) (28), is phosphorylated in response to growth factors acting through receptor tyrosine kinases (29) and integrin-mediated signaling (30). As Cas has an SH3-domain as well as multiple SH2-binding motifs, Cas may well assemble a number of proteins such as FAK (20) and the phosphatases PTP-PEST (31) and PTP1B (32) into a single complex. Cas may be a critical component by which extracellular events influence cell morphology and survival (33–35).

A theme running through the mechanism by which transcription factors such as AP-1 are activated by receptor tyrosine kinases and integrin receptors is the importance of adaptor proteins. These adaptor proteins contain one or multiple domains that mediate protein-protein or protein-lipid interactions (for review, see Ref. 36). In addition to integrating independent extracellular signals, cell type-specific expression of the adaptor molecules may be critical in determining the cell type-specific response to extracellular stimuli. Although most of the more extensively characterized signal transduction molecules are ubiquitously expressed (15, 37, 38), a few adaptor

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‡ The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEKK, MAPK/ERK kinase kinase; JNK, c-Jun N-terminal kinase; EGF, epidermal growth factor; FAK, focal adhesion kinase; EST, expressed sequence tag; CHO, Chinese hamster ovary; SRE, serum response element.

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10047
molecules with a limited pattern of expression have been identified, such as N-She (37) and Grb-IR (39).

To identify additional adapter proteins that are involved in growth factor and integrin signaling, we have searched an expressed sequence tag (EST) database for sequences that share homology with SH2-containing adapter proteins. By this method, we isolated a cDNA encoding a family of novel SH2-containing proteins. The structure and characteristics of at least NSP1 (novel SH2-containing protein 1) suggest that this protein may play an important role in cell proliferation and fetal development.

**EXPERIMENTAL PROCEDURES**

**Cloning of NSP1, NSP2, and NSP3 cDNAs**—A proprietary EST database (Incyte) was screened for sequences that encode homologues of proteins known to be involved in intracellular signaling. One EST from a fetal pancreas library encoded an SH2 domain related to human SHC. A full-length cDNA encoding NSP1 was cloned from a human fetal kidney cDNA plasmid library in pRK vector using standard polymerase chain reaction and hybridization protocols. The full-length NSP1 cDNA sequence was then used to resequence the EST database. Two related cDNAs, NSP2 and NSP3, were subsequently cloned. The Flag epitope (DYKDDDDK) was added in frame to the N terminus of the NSP1 cDNA construct using in vitro mutagenesis (Stratagen) to create pRK.NSP1.Flag. All three tyrosine residues in NSP1 were independently mutated to phenylalanine (Y16F, Y95F, and Y231F) using in vitro mutagenesis.

**Northern Hybridization Analysis**—The human multiple-tissue Northern I, fetal II, and human immune system II blots (Clontech) were hybridized with a 32P-random primer-labeled NSP probe representing the SH2 domain and a 3'end-labeled oligonucleotide NSP2 probe corresponding to amino acids 270–284 and NSP3 probe corresponding to amino acids 475–491 according to the manufacturer’s instructions.

**Cell Culture and Transfection**—COS and 293 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C. CHO-IR cells (CHO cells overexpressing insulin receptor, provided by Craig Crowley, Genentech, Inc.) and CHO cells were cultured in nutrient mixture F-12/Dulbecco’s modified Eagle’s medium supplemented as described above. Liposome-mediated transfections using DOSPER (Boehringer Mannheim) or Superfect (QIAGEN Inc.) were carried out on CHO, COS, or 293 cells in accordance with the manufacturers’ instructions.

**Immunoprecipitation and Western Blot Analysis**—Transiently transfected cells were lysed on ice for 1 h in 1 ml of immunoprecipitation assay buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 2 mM EDTA 10 mM sodium pyrophosphate, 10 mM sodium fluoride, and 2 mM orthovanadate) containing freshly added protease inhibitors (1 mM (2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml aprotinin, 2 μg/ml aprotinin, and 1 μM pepstatin). Samples were immunoprecipitated with anti-Flag affinity gel (Kodak Scientific Imaging Systems), anti-EGF receptor antibody (Calbiochem), or anti-c-Myc antibody (Boehringer Mannheim). The immunoprecipitates were subjected to either Western blotting or an in vitro kinase assay. For Western blotting, following SDS-polyacrylamide gel electrophoresis, proteins were transferred onto nitrocellulose membrane (Novex) and analyzed using the anti-phospho-tyrosine antibody PY20 (Transduction Laboratories), or the anti-p130Cas antibody (Fig. 4A). The tyrosine-phosphorylated protein subsequently identified as p130Cas (Cas) using an anti-Flag antibody (Fig. 4A) corresponds to amino acids 270–284 and 475–491 according to the manufacturer’s instructions.

**RESULTS**

**cDNA Cloning of NSP1, NSP2, and NSP3**—A proprietary EST database was searched for sequences related to signal transduction motifs. An EST homologous to the SH2 domain of the adaptor protein Shc was found and used to clone a corresponding full-length cDNA encoding a 576-amino acid protein: NSP1. NSP1 is most closely related to Shc (34% identity over a 92-amino acid region in the Shc SH2 domain), although this similarity only covers the SH2 region that appears at the N terminus of NSP1 (Fig. 1). NSP1 also contains a proline/serine-rich domain (PS domain) that may function as an SH3 interaction domain. Rescreening the EST database with the NSP1 sequence allowed the cloning of two related cDNAs (NSP2 and NSP3). These three proteins (calculated molecular masses of 63.1, 92.6, and 77.1 kDa) have an overall shared identity of between 25 and 39%, and all three proteins contain SH2 domains and potential SH3 interaction domains. None of the three NSP proteins contain obvious kinase or phosphatase domains, but all three do contain an amino acid motif that is consistently found in proteins with guanylate exchange activity. Whether there is a guanylate exchange activity associated with these proteins is being investigated.

**Expression of NSP1, NSP2, and NSP3 in Human Tissues**—By Northern hybridization analysis, NSP1 is weakly expressed in comparison to NSP2 and NSP3, and significant levels of NSP1 mRNA were only detected in the placenta; fetal kidney and lung; and adult pancreas, kidney, and lung (Fig. 2A). Whether there is a biological relevance to the apparent specificity of expression in organs enriched in secretory epithelial cells is of some interest. In addition, we have detected expression of NSP1 in some cell lines including SW480 (data not shown). In contrast, NSP2 and NSP3 are expressed in a wide variety of fetal and adult tissues. In hematopoietic tissues, two NSP3 transcripts were detected (Fig. 2B). The relative level of the two transcripts in these hematopoietic tissues does vary; some such as the thymus express only the higher molecular mass form.

**NSP1 Phosphorylation by EGF and Insulin**—As NSP1 contains three tyrosines, we investigated whether NSP1 could be phosphorylated in response to extracellular stimuli. COS and CHO-IR cells were used to investigate responses to EGF and insulin, respectively. Cell extracts were prepared following transfection with Flag-tagged NSP1 expression plasmids, and NSP1 was immunoprecipitated with anti-Flag affinity gel and Western-blotted with an anti-phosphotyrosine antibody. Treatment with either insulin or EGF for 10 min induced rapid tyrosine phosphorylation of NSP1 (Fig. 3).

**Complex Formation of NSP1 with p130Cas and the EGF Receptor**—Co-immunoprecipitation experiments were performed to identify protein partners of NSP1. Under basal conditions (serum-starved cells attached to tissue culture plates or cells held in suspension for 30 min), immunoprecipitated, unphosphorylated NSP1 was found to interact with a 130-kDa protein subsequently identified as p130Cas (Cas) using an anti-Cas antibody (Fig. 4A). The tyrosine-phosphorylated protein on Western blots of NSP1 with a molecular mass of ~120 kDa has not yet been identified. EGF increased NSP1 phosphorylation, but led to a decrease in the complexity of the Cas that was associated with NSP1 (Fig. 4A, upper panel). The apparent decrease in Cas phosphorylation becomes more significant when the EGF-stimulated increase in the amount of Cas that is immunoprecipitated is taken into account (Fig. 4A, center panel). Insulin treatment of CHO-IR cells induced comparable changes (data not shown). In contrast to the response to receptor tyrosine kinases, rephosphorylation of de-
attached cells on fibronectin stimulated only weak NSP1 phosphorylation (Fig. 4B), but led to a significant increase in the phosphorylation of the Cas that was associated with NSP1 and, at the same time, a transient dissociation in the amount of Cas that was associated with NSP1. The amount of the NSP1 Cas complex reached a nadir at 30 min following contact with the fibronectin and then returned toward baseline conditions at ~4 h. The band below NSP1 appears to be nonspecific.

In COS cells treated with EGF, a 170-kDa protein was co-immunoprecipitated with NSP1 (Fig. 4A). This protein was rapidly tyrosine-phosphorylated in response to EGF and could be detected with an antibody against the EGF receptor (data not shown). In a reverse experiment, we showed that NSP1 could be detected following immunoprecipitation of the EGF receptor and that the extent of the interaction significantly increased following exposure to EGF (Fig. 5). Under these conditions, we could detect EGF receptor-associated NSP1 using the anti-Flag antibody (Fig. 5, upper panel), but could not detect phosphorylated NSP1 using the anti-phosphotyrosine antibodies. It is unclear whether this is a technical issue (e.g., relative affinities of the two antibodies) or reflects the state of the NSP1 that remains associated with the receptor.

Identification of Phosphorylated Tyrosine Residues in NSP1—To determine which tyrosine residues in NSP1 are phosphorylated, we changed each of the three tyrosines in NSP1 to phenylalanine. Transfected cells were then stimulated with EGF for 10 min, and NSP1 was immunoprecipitated (Fig. 6). Mutant NSP1Y16F was phosphorylated normally in response to EGF, suggesting that Tyr-16 may not be phosphorylated. However, there was no detectable phosphorylation of NSP1Y95F, and NSP1Y231F was weakly phosphorylated. Note that each of these variants can still interact with the EGF receptor whether or not NSP1 is phosphorylated, demonstrating that the amino acid substitution does not lead to gross protein misfolding and that the lack of phosphorylation is not simply the result of failure to interact with the EGF receptor. Although other possibilities exist, these data suggest a sequential phosphorylation model in which there is an obligatory first phosphorylation event at Tyr-16 before phosphorylation at Tyr-95.

![Figure 1](image1.png)

**FIG. 1.** Structural characteristics and alignment of NSP1, NSP2, and NSP3. The amino acid sequences and the alignment of NSP1, NSP2, and NSP3 deduced from the cloned cDNAs are shown. The approximate delimitation of the SH2 domains and the proline/serine (PS)-rich regions are indicated and shaded in gray.
phosphorylation of Tyr-95, followed by the phosphorylation of Tyr-231.

**NSP1 Activates the JNK1 Kinase and AP-1**—To identify downstream effects of NSP1, 293 cells were cotransfected with NSP1 and the expression plasmids for JNK1 or ERK2. The kinases were immunoprecipitated, and their activities were determined with recombinant ATF-2 or myelin basic protein as a substrate in vitro kinase assays. Although there was no apparent increase in ERK2 kinase activity (Fig. 7A), increased expression of NSP1 led to a dose-dependent increase in JNK1 activity (Fig. 7B). Activation of JNK should lead to phosphorylation of c-Jun and the subsequent increase in expression from reporter genes under the control of AP-1 recognition sequences. Conversely, the failure to activate ERK should be revealed by a lack of activation of promoters containing a serum response element (SRE). Thus, NSP1 was cotransfected with the luciferase cDNA controlled by a promoter containing either AP-1 or SRE interaction sites. Consistent with the results of the in vitro kinase assays, we have found that NSP1 expression led to a 7-fold increase in the level of expression of an AP-1-luciferase gene. Under the same conditions, there was no increase in expression of the luciferase gene containing SRE recognition sequences (Fig. 8). In both experiments, transfection with a plasmid expressing the MAPK kinase MEKK led to the expected increase in luciferase expression.

**DISCUSSION**

We have isolated a family of novel adaptor proteins (NSP1, NSP2, and NSP3) that contain an N-terminal SH2 domain, a central proline/serine-rich region, and a C-terminal sequence that is distinctive for the NSP proteins. While this manuscript was in preparation, the sequence of NSP2 was published as BCAR3; this protein (BCAR3) appears to be implicated in the development of resistance to the cytostatic agent Taxol (40).
Increased expression of NSP1 results in JNK (but not ERK) activation. 293 cells were transfected with 2.5 μg of pJNK-Myc (A) or pRK.ERK2.Flag (B) and increasing amounts of NSP1 expression plasmids as indicated. The anti-Flag or anti-c-Myc immunoprecipitates were evaluated for their ability to phosphorylate myelin basic protein (MBP: A) or ATF-2 (B) in an in vitro kinase assay (JNK). The same blots were used in Western blotting (W) to show the amount of JNK1 and ERK2 proteins in each lane. IP, immunoprecipitation.

NSP1 defines a novel family of adaptor proteins

Increased expression of NSP1 leads to the activation of the AP-1 (but not SRE) promoter. CHO cells were cotransfected with 0.5 μg of the pAP-1-Luc or pSRE-Luc reporter plasmid and 2 μg of the indicated NSP1 expression plasmids. The relative luciferase activities were generated using Renilla luciferase (see “Experimental Procedures”). Each point is the average of triplicates. The results represent one of three replicates.

NSP1 expression may be restricted to tissues with secretory epithelial cells, whereas NSP2 and NSP3 are expressed in variety of tissues. In hematopoietic tissues, two NSP3-related transcripts are detected. The coding potential of these two NSP3 transcripts is being explored.

NSP1 is phosphorylated in response to insulin and EGF (and insulin-like growth factor I and heregulin (data not shown)), indicating that NSP1 is a common target for a variety of growth factor receptors. In response to EGF, NSP1 associates with the EGF receptor and is phosphorylated. The data are consistent with a direct interaction between the phosphotyrosines on the EGF receptor and the NSP1 SH2 domain and a subsequent receptor kinase-dependent phosphorylation of NSP1. Preliminary data indicate that the SH2 domain alone is able to interact with the EGF receptor, although other regions of NSP1 may also participate in this interaction (data not shown). It is also possible that the EGF receptor-NSP1 interaction is indirect. NSP1 is only modestly phosphorylated in response to integrin signaling. This weak integrin-mediated phosphorylation could conceivably be through FAK or Src-related kinases, both of which are known to associate with Cas (20, 22, 25, 28, 41).

Co-immunoprecipitation experiments revealed that NSP1 interacts with p130Cas. Preliminary data from a yeast two-hybrid analysis are consistent with this result and suggest that the interaction is direct (data not shown). In the co-immunoprecipitation experiments, the interaction between NSP1 and Cas could be detected under conditions in which there was no detectable phosphorylation of NSP1 or Cas, suggesting that the interaction between NSP1 and Cas is phosphorylation-independent and may occur via the SH3 domain in Cas and the proline/serine-rich domain in NSP1. Neither of the demonstrated associations (EGF receptor and Cas) appears to utilize the phosphotyrosines in NSP1. This conclusion is reinforced by the experiments using the tyrosine replacement variants in which both EGF receptor and Cas interactions can occur in the absence of any detectable NSP1 phosphorylation.

The relative level of the NSP1-Cas complex and the phosphorylation status of Cas are quite different between receptor tyrosine kinase and integrin signaling. Thus, EGF treatment leads to NSP1 phosphorylation, to dephosphorylation of the Cas that is associated with NSP1, and to an increase in the amount of the NSP-Cas complex. In contrast, in response to fibronectin, there is little change in NSP1 phosphorylation, but there is a significant increase in the phosphorylation of the Cas associated with NSP1. Cas dephosphorylation in response to EGF (42) and Cas phosphorylation in response to fibronectin (30, 30, 43, 44) have been previously reported. There is also an apparent dissociation of NSP1 from Cas at short time periods and a subsequent reassociation at longer (4 h) times. These results led to the hypothesis that the biological outcome in response to extracellular signals could be quite distinct in the presence or absence of NSP1. For example, FAK associates with the SH3 region in Cas via a Proline/serine-rich domain in NSP1. Neither of the demonstrated interactions (NSP1-Cas and EGF-Cas) appear to utilize the NSP1 phosphotyrosines in NSP1. Thus, it is possible that NSP1 could compete for the SH3 region in Cas and so alter FAK-dependent events (21). In contrast, EGF treatment leads to NSP1 phosphorylation, to dephosphorylation of NSP1-associated Cas, and to an increase in the amount of the NSP1-Cas complex. This complex is then likely to have a decrease in the number of proteins associated with the phosphotyrosines in Cas and so may lead to changes in downstream signaling.

Increased expression of NSP1 results in JNK activation and increased expression from an AP-1-dependent promoter. Whether this activation of JNK is related to the EGF-stimulated phosphorylation of NSP1 or the interaction of Cas with NSP1 is currently unknown, although preliminary data from a luciferase assay indicate that NSP1_P715SRP, which is not phosphorylated but still interacts with both Cas and the EGF receptor, has diminished but not abolished activity in this assay. It has been previously shown that activation of the JNK kinase cas-
caden is dependent on one of several small GTP-binding proteins (Cdc42, Rac, and Ras) (12, 14) and on phosphatidylinositol 3-kinase (19). How or whether NSP1 can modify any of these components of the signaling pathway is under investigation.

The activation of the c-Jun kinases in response to receptor tyrosine kinase and integrin receptor signaling appears to be critical for regulating cell proliferation (45–47). Furthermore, genetic analysis of JNK signaling in Drosophila (5) and the abnormal liver development in mice genetically lacking the JNK kinase activator MKK4 suggest a critical role for JNK in normal development (48). Thus, the identification of a novel adaptor protein that functions in these processes may provide a valuable molecular tool for understanding cell proliferation and fetal development.

Acknowledgments—We thank L. Holzman for the JNK-Myc expression construct and Wayne Anstine and Louis Tamayo for the graphics.

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