The Prooncoprotein EWS Binds Calmodulin and Is Phosphorylated by Protein Kinase C through an IQ Domain*  

Jean Christophe Deloulme‡§, Lisa Prichard‡, Olivier Delattre¶, and Daniel R. Storm‡‡  

From the 3University of Washington, Department of Pharmacology, Seattle, Washington 98195 and ‡INSERM U 434, Génétique des Tumeurs, Institut Curie, 26 rue d’Ulm, 75231 Paris Cedex 05, France

We report that EWS, a nuclear RNA-binding prooncoprotein, contains an IQ domain, is phosphorylated by protein kinase C, and interacts with calmodulin. Interestingly, PKC phosphorylation of EWS inhibits its binding to RNA homopolymers, and conversely, RNA binding to EWS interferes with PKC phosphorylation. Several other RNA-binding proteins, including TLS/FUS and PSF, co-purify with EWS. PKC phosphorylation of these proteins also inhibits their binding to RNA in vitro. These data suggest that PKC may regulate interactions of EWS and other RNA-binding proteins with their RNA targets and that IQ domains may provide a regulatory link between Ca2+ signal transduction pathways and RNA processing.

Neuromodulin (GAP-43, B-50, F1) and neurogranin (RC3, BICKS) are neurospecific calmodulin (CaM)1-binding proteins that are phosphorylated by protein kinase C (PKC) (for reviews see Refs. 8 and 42). These proteins share a nearly identical sequence of 20 amino acids designated the IQ motif containing a CaM-binding domain and a PKC phosphorylation site (1–4). The interaction between CaM and the IQ domain of neuromodulin or neurogranin has been characterized extensively in vitro (2, 5–9). The affinity of neuromodulin or neurogranin for CaM is higher in the absence than in the presence of Ca2+, and CaM binding is inhibited by PKC phosphorylation within the IQ domain. Furthermore, CaM binding inhibits PKC phosphorylation of neuromodulin and neurogranin at a specific serine in the IQ domain. These observations led to the hypothesis that neuromodulin may bind and concentrate CaM at specific sites in neurons and release CaM in response to PKC phosphorylation or increases in Ca2+ (5, 10). Recent data from several laboratories have confirmed that neuromodulin and CaM interact in cells. For example, elevation of free Ca2+ or PKC activation in primary hippocampal neuron cultures inhibits CaM/neuromodulin interactions in vivo (11). Neuromodulin and CaM have also been shown to interact in vivo using the yeast two-hybrid system (12).

The IQ domain may serve as a general regulatory domain in proteins for CaM binding and PKC phosphorylation. It has been found in other proteins including the Ca2+-vector protein target from amphioxus (13), the neurospecific peptide PEP-19 (14), the early endosome-associated protein EEA1 (15) and the guanine nucleotide exchange factor p140/Ras-GRF (16). The conventional and unconventional myosins (17), the igloo protein from Drosophila (18), the GTPase-activating protein IQ-GAP (19, 20), and the docking protein of insulin receptor IRS-1 (21) all contain repeat IQ motifs. Some of these proteins, e.g. unconventional myosins and PEP-19 (14), interact with CaM in a Ca2+-independent manner, whereas the Ca2+-vector protein target and p140/Ras-GRF bind CaM in the presence of Ca2+ (16, 22). The IQ domain may also mediate interactions with other Ca2+-binding proteins belonging to the EF-hand family. The repeat IQ motifs of conventional myosins bind myosin light chains (23), and neuromodulin and neurogranin can also interact with the S100b protein (24, 25). The conservation of the IQ regulatory sequence in a variety of proteins strongly suggests that the molecular function of these proteins may be regulated by Ca2+-signal transduction pathways through IQ domain(s), although these proteins have very different biochemical functions.

Using an antibody against the IQ domain of neurogranin, we discovered a new member of the IQ protein family, P68-RNA helicase, the first nuclear IQ domain protein identified (26). Human P68-RNA helicase is an RNA-dependent ATPase that belongs to the family of putative helicases known as the DEAD box proteins (27). These proteins are implicated in some aspects of RNA functions including translation initiation, splicing, and ribosome assembly. PKC phosphorylation and CaM binding both inhibit P68-RNA helicase ATPase activity, suggesting that its RNA unwinding activity or binding to RNA may be regulated by Ca2+-signal transduction pathways (26). Other lines of evidence suggest that RNA processing may be controlled by Ca2+-signal pathways. Recently, Bachs and colleagues (28) discovered that heterogeneous nuclear ribonucleoproteins (hnRNPs) A2 and C interact with CaM in the presence of Ca2+. Furthermore, hnRNP A1 is phosphorylated in vivo by PKC-γ (29), and PKC phosphorylation of hnRNP A1 regulates its RNA binding activity in vitro (29, 30).

Since CaM is also present in the nucleus (31) and P68-RNA helicase contains an IQ domain, we examined nuclear extracts for other RNA-binding proteins containing IQ domains. We found that the human nuclear RNA-binding prooncoprotein EWS (Ewing sarcoma protein) contains an IQ domain, binds to...
CaM, and is phosphorylated by PKC. The EWS gene is implicated in specific chromosomal translocations occurring in peripheral primitive neuroectodermal tumors (32, 33). We also report that EWS co-purifies with another RNA-binding protein and PKC substrate, TLS/FUS (34–36) as well as the splicing factor PSF (37). Interestingly, PKC phosphorylation of EWS, TLS/FUS, and PSF inhibits their RNA binding activity in vitro.

**EXPERIMENTAL PROCEDURES**

**Materials**—The alkaline phosphatase-conjugated substrate kit and prestained SDS-PAGE standards were from Bio-Rad. The nitrocellulose membrane was from Schleicher and Schuell. The polyvinylidene difluoride membrane for protein sequencing (Immobilon P™) was from Millipore Corp. (Bedford, MA). High resolution hydroxyapatite resin was from Calbiochem. Fibrous cellulose CF11 was from Whatman. Sequencing grade trypsin and V8 protease were from Promega (Madison, WI). ssDNA-cellulose, dsDNA-cellulose, CaM-agarose, l-a-phosphatidyl-serine, and diolein were from Sigma. CNBr-activated Sepharose 4B and ribonucleotide homopolymers were from Pharmacia Biotech Inc. [γ-32P]ATP (3000 Ci/mmol) was from NEN Life Science Products. Dulbecco’s modified Eagle’s medium, bovine calf serum, fetal calf serum, and biotinylated calmodulin were from Life Technologies, Inc. PKC was purified from rabbit brains as described by Alexander et al. (5) and was purchased from Boehringer Mannheim.

**Protease Inhibitors**—Leupeptin, aprostatin, pepstatin A, and phenylmethylsulfonyl fluoride were purchased from Sigma and used at final concentrations of 5 μg/ml, 10 μg/ml, 5 μM, and 1 mM, respectively. The addition of protease inhibitors in solutions and buffers is indicated as mixture inhibitor.

**Cultured Cells**—Human embryonic kidney 293 (HEK-293) cells and mouse fibroblast 3T3-L1 cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and 5% bovine calf serum in a humidified 95% air and 5% CO2 incubator.

**Isolation of Subcellular Fractions**—All extraction steps were performed at 4 °C. HEK-293 or 3T3-L1 cells (100-mm plates) were washed twice with 3 °C phosphate-buffered saline (PBS), scraped off, and collected by centrifugation for 5 min at 200 × g. Pelleted cells were suspended in 5 volumes of hypotonic buffer containing 10 mM Tris-HCl, pH 6.8, at 25 °C, 0.5 mM DTT, 100 mM NaCl, 2.5 mM MgCl2, 0.5% Triton X-100, and mixture inhibitor. The membrane fraction was clarified by centrifugation for 5 min at 200 × g. Pelleted cells were washed with 4 °C phosphate-buffered saline (PBS), scraped off, and collected by centrifugation for 5 min at 200 × g. Pelleted cells were then vigorously vortexed and incubated with 4 °CPBS, 0.5% Triton X-100, and mixture inhibitor. The supernatant was collected by centrifugation for 5 min at 200 × g.

**Protein Phosphorylation Assays**—The PKC phosphorylation assays were carried out as described by Alexander et al. (5). The type A or B fraction (1 μl, 40 μg/ml) was incubated with purified PKC in 20 mM Hepes, pH 7.4, 5 mM MgCl2, 5 mM DTTP in the presence or absence of 1.5 mM CaCl2, 60 μg/ml l-a-phosphatidyl-serine, 6 μg/ml diolein, and 70 mM bisindolylmaleimide for the indicated times at 30 °C. The reaction was initiated by the addition of 500 μl [γ-32P]ATP. The total volume of the reaction was 50 μl, and the reaction was terminated by the addition of Laemmli sample buffer or urea. The proteins were separated by 12% SDS-PAGE and the gels were stained with Blue, dried, and autoradiographed. The autoradiograms were scanned with a Scan Jet II CX scanner and analyzed by densitometry using NIH Image software.

**Protein Cleavage and Amino Acid Sequencing**—Peptide mapping was carried out as described by Cleveland et al. (39), with some modifications. Briefly, the type A fraction (five lanes loaded with 40 μg) was run on 8% SDS-PAGE and stained with Coomassie Blue. The band corresponding to IQ65 was excised from the gel and electrophoresed on a 12% SDS-PAGE gel in the presence of trypsin (0.5 μg/ml, 0.125 mM Tris-HCl, pH 7.8, at 25 °C, 1 mM MgCl2, and 0.1% SDS). To increase the efficiency of cleavage by trypsin, the pH of the stacking gel was brought to 7.8, and 1 mM CaCl2 was added. Digested peptides were electrophoretically transferred to a polyvinylidene difluoride membrane and stained with Coomassie Blue. Selected peptide fragments were sequenced with an automated amino acid sequencer (Applied Biosystems, model 477A).

**Western Blots**—Proteins were electrophoresed by SDS-PAGE and transferred electrophoretically to nitrocellulose membrane as described by Towbin et al. (40). After transfer, the membrane was blocked for 1 h with 3% cold fish gelatin in PBS buffer. After washing in PBS, the blot was incubated overnight at 4 °C with primary antibodies in TPBS (PBS plus 0.05% Tween 20) as indicated. Bound antibody was detected using alkaline phosphatase-conjugated secondary antibody and an alkaline phosphatase-conjugated substrate kit according to the manufacturer’s instructions.

**Immunoprecipitation**—Flow-through fractions from a hydroxyapatite column (100 μl) were precleared with 20 μl of protein A-Sepharose beads. The supernatant was incubated with IgG antibody (2 μg of IgG) or EWS 677 antibody (10 μl) overnight with agitation, and then 20 μl of protein A-Sepharose beads was added for 1 h. Beads were pelleted and washed with PBS plus 1% Triton X-100 (5 × 1 ml). Immunoprecipitated proteins were analyzed by Western blot using EWS and IQ antibodies.

**Other Methods**—SDS-PAGE was performed as described by Laemmli et al. (41), and proteins were quantified by the Bradford method (42). The BCA protein assay kit (Pierce) was used to determine the concentration of neurogranin and neuromodulin. CaM binding was monitored by biotinylated calmodulin overlay (43).
Inhibition of EWS RNA Binding by PKC Phosphorylation

**RESULTS**

**Characterization of IQ85**—To identify nuclear proteins that contain IQ domains, we performed Western analysis of subcellular fractions from HEK-293 and mouse 3T3-L1 fibroblast cells using a polyclonal antibody (IQ Ab) that recognizes the IQ domain of neuromodulin, neurogranin, and P68-RNA helicase (26). In both cell lines, the IQ Ab recognized P68-RNA helicase and a larger protein of 85 kDa (Fig. 1A). An additional protein with an apparent molecular mass of 100 kDa was detected in the HEK-293 cell nuclear extracts. These three proteins were localized in the nuclear extract (NE) but were not found in cytoplasmic (Ct) or membrane fractions (Mb). To determine if the 85- and 100-kDa nuclear proteins contained IQ domains, the IQ Ab was incubated with an excess of pepP68, a peptide containing the IQ domain of P68-RNA helicase (P68). Standard molecular masses (in kDa) are indicated to the right and the positions of P100, P68-RNA helicase, and IQ85 are shown to the left of the blots.

Bach and Carafoli (31) reported that CaM is associated with the nuclear matrix fraction that contains nuclear membrane proteins, proteins implicated in DNA transcription and replication, and proteins involved in RNA metabolism (44). Furthermore, P68-RNA helicase may also be associated with the nuclear matrix (45). Therefore, we used the IQ Ab and a P68-RNA helicase monoclonal antibody, PAb204 (46), to investigate the subnuclear localization of IQ85 and P68-RNA helicase in HEK-293 cell nuclei. Purified nuclei were treated with DNase I and RNase A, followed by solubilization using 0.5% Triton X-100 and 1.5 mM NaCl. The solubilized proteins (S1) and the pellet containing the nuclear matrix fraction (Mx) were analyzed for IQ domain-associated proteins. Western blot analysis with PAb204 and IQ Ab, which both recognize P68-RNA helicase (P68), standard molecular masses (in kDa) are indicated to the right and the positions of P100, P68-RNA helicase, and IQ85 are shown to the left of the blots.

Partial Purification of IQ85—To identify IQ85, fractions from purified nuclei of quiescent HEK-293 cells were prepared as described under “Experimental Procedures,” and IQ85 was partially purified. The first purification step was similar to that used for isolation of P68-RNA helicase (45). An alkaline buffer was used to extract protein from the nuclear matrix, which was then applied to a hydroxyapatite column. Hydroxylapatite fractions were analyzed on 8% SDS-PAGE by Coomassie Blue staining (Fig. 2A) and immunoblotting with the IQ Ab (Fig. 2B). Unlike P68-RNA helicase, which absorbed to the column and was concentrated in the eluate (Ei), IQ85 was not retained on hydroxylapatite and was found exclusively in the flowthrough (FT) fraction (Fig. 2B). Additional purification of IQ85 was achieved by ammonium sulfate (AS) precipitation of the hydroxylapatite FT fraction. IQ85 was one of the major Coomassie Blue-stained proteins in this fraction (Fig. 2A). The ammonium sulfate pellets were insoluble in buffers lacking detergents and could only be resuspended in the presence of 1% SDS or 2 mM urea, suggesting that the precipitated proteins were strongly aggregated. We obtained two types of ammonium sulfate fractions, depending upon the number of HEK-293 cell passages (Fig. 2C). The type A fraction was obtained from HEK-293 cells grown less than 10 passages, whereas the type B fraction was obtained when cell passage was greater than 10. The type A fraction contained five major Coomassie Blue-stained proteins designated P98, IQ85, P70, P60, and P48. P98 and P60 were absent from type B preparations. Densitometry analysis of Coomassie Blue-stained gels showed that IQ85 represented about 20% and 40% of total protein in type A and type B fractions, respectively.

Bacterial and rabbit reticulocyte polypeptide extracts were run on 8% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with the IQ domain antibody. B, IQ85 contains an IQ epitope. Western blot analysis of HEK-293 cell nuclear extract (lanes NE, 15 μg) using the IQ Ab in the absence or presence of 0.3 mg/ml pepP68. C, IQ85 and P68-RNA helicase are present in the nuclear matrix. Extraction of nuclear matrix-associated proteins (lanes Mx, 10 μg) after treatment of HEK-293 nuclei with RNase A and DNase I, 1.5 mM NaCl, and 0.5% Triton X-100 (lanes S1, 10 μg) was performed as detailed under "Experimental Procedures." Samples were analyzed by Western blot using the IQ Ab and a P68-RNA helicase monoclonal antibody, PAb204 (46), to investigate the IQ85 position of P100, P68-RNA helicase, and IQ85 are shown to the left of the blots.

**Fig. 1. Expression and subcellular localization of IQ85.** A, IQ85 is a nuclear protein. Nuclear extract from HEK-293 cells and nuclear extract (lanes NE, 15 μg), cytosolic fraction (lane Ct, 15 μg), and membrane fraction (lane Mb, 15 μg) from 3T3-L1 cells were separated by 8% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with the IQ domain antibody. B, IQ85 contains an IQ epitope. Western blot analysis of HEK-293 cell nuclear extract (lanes NE, 15 μg) using the IQ Ab in the absence or presence of 0.3 mg/ml pepP68. C, IQ85 and P68-RNA helicase are present in the nuclear matrix. Extraction of nuclear matrix-associated proteins (lanes Mx, 10 μg) after treatment of HEK-293 nuclei with RNase A and DNase I, 1.5 mM NaCl, and 0.5% Triton X-100 (lanes S1, 10 μg) was performed as detailed under “Experimental Procedures.” Samples were analyzed by Western blot using the IQ Ab and a P68-RNA helicase monoclonal antibody, PAb204 (46), to investigate the IQ85 position of P100, P68-RNA helicase, and IQ85 are shown to the left of the blots.

**Fig. 2. Partial purification of IQ85.** IQ85 was partially purified from HEK-293 nuclear extract as described under “Experimental Procedures.” Samples from the load (lanes S100, 15 μg), flow-through fraction (lanes FT, 15 μg), elution fraction (lanes Ei, 15 μg), and ammonium sulfate pellet (lanes AS, 5 μg) were run on 8% SDS-PAGE, stained with Coomassie Blue (A), or transferred and immunoblotted with the IQ Ab (B). Molecular weight standards, IQ85, and P68 are indicated beside the blots. C, type A (6 μg) and type B (3 μg) ammonium sulfate pellets were analyzed by 8% SDS-PAGE. The major Coomassie Blue-stained proteins are indicated to the left, and molecular weight standards are indicated to the right.
In vitro RNA binding activity of IQ85 and co-purifying proteins. Chromatography on a ssDNA-cellulose column (A) and a dsDNA-cellulose column (B) was performed in the presence of 2 M urea. Loaded type A fractions (lane Ld. Type A, 6 µg), flow-through fractions 2 and 3 (lanes FT, 35 µl), and eluted fractions 2 and 3 (lanes Hep, lanes NaCl 0.5 M, lanes NaCl 1 M, 35 µl) were analyzed by 8% SDS-PAGE and stained with Coomassie Blue. C. RNA-binding of IQ85 and co-purifying proteins was performed by incubation of the type A fraction with poly(U) (lane u), poly(G) (lane g), poly(A) (lane a), and poly C (lane c) homopolymer-Sepharose beads. Beads were washed extensively in urea buffer, drained, and boiled for 5 min in Laemmli buffer (40 µl). Bound proteins were subjected to 8% SDS-PAGE and stained with Coomassie Blue. IQ85 and co-purifying proteins are identified to the left.

Identification of IQ85, P98, P71, and P60—To identify IQ85, the protein was subjected to peptide mapping using a modified Cleveland method (39). We sequenced three tryptic fragments from IQ85 (Fig. 4A), which corresponded exactly to sequences (32) found in human EWS. To confirm the sequencing data, IQ85 was immunoprecipitated with an antibody raised against the N-terminal domain of EWS (EWS 677 Ab), and Western analysis was carried out using the IQ Ab. The IQ Ab recognized the protein immunoprecipitated by the EWS antibody (Fig. 4B). Conversely, IQ85 immunoprecipitated with IQ Ab was also recognized by the EWS antibody. These data indicated that IQ85 is the prooncoprotein EWS, a putative RNA-binding protein expressed in normal cells (32, 47, 48). To identify other RNA-binding proteins that co-purified with EWS, we sequenced three tryptic peptides and one V8 peptide from IQ85 (Fig. 4A) and compared the corresponding sequences found in the human EWS and PSF sequences, respectively. B, hydroxylapatite column flow-through fractions were immunoprecipitated with the EWS 677 Ab (lane EWS 677 Ab IP) or with the IQ domain antibody (lane IQ Ab IP). Controls were performed by omitting primary antibodies during the immunoprecipitation (lanes Control IP). Immunoprecipitated fractions and HEK-293 nuclear extract (lanes NE, 15 µg) were subjected to Western blot analysis using the IQ domain Ab (left) and the EWS 677 Ab (right). The large band at 55 kDa (lanes EWS 677 IP and IQ Ab IP) corresponds to the IgG heavy chain of EWS 677 Ab and IQ Ab. C, Western blot analysis of the type A fraction (lanes A, 4 µg), type B fraction (lanes B, 2 µg), and HEK-293 nuclear extract (lanes NE, 15 µg) using sera raised against PSF (PSF Ab), p54+/−, and TLS/FUS (TLS Ra). The positions of PSF/P98, TLS/FUS/P71, EWS/IQ85, and p54+/−/P60, are shown to the left.

Localization of the IQ Epitope within the EWS Amino Acid Sequence—To identify the EWS IQ domain, we compared the amino acid sequences of EWS with neurogranin, P68-RNA helicase, and neuromodulin. The sequence alignment of neurogranin, P68-RNA helicase, and EWS with the IQ domain of neurogranin shows that the 19 IQ domain residues from neurogranin-(27–45), 13 are present in neuromodulin-(34–52), 6 in P68-RNA helicase-(550–568), and 4 in EWS-(259–277) (Fig. 5A, boldface type). The sequence QXSFR contains the four amino acids common to all of these proteins. In neuromodulin and neurogranin, the serine within this sequence has been identified as the PKC phosphorylation site (Fig. 5A, asterisk). To determine if the IQ Ab recognizes EWS through the IQ domain described above (EWS-(259–277)), we synthesized a peptide (pepEWS) corresponding to this sequence (Fig. 4A). We monitored the ability of the peptides pepEWS and pepP68 to suppress the immunoreactivity of the IQ Ab toward EWS, P68-RNA helicase, neuromodulin, and GST-neurogranin (GNg). An excess of pepP68 or pepEWS was preincubated with the IQ Ab prior to Western blotting. These peptides completely suppressed the reactivity of the IQ Ab toward EWS and P68-
Inhibition of EWS RNA Binding by PKC Phosphorylation

RNA helicase from HEK-293 nuclear extract (NE), EWS from the type B fraction, and purified rat neuromodulin (Fig. 5B). PepEWS and pepP68-RNA helicase also partially decreased the immunoreactivity of the IQ domain antibody against GST-neurogranin fusion protein (NGn), and neuromodulin (Nm) are indicated to the left.

Phosphorylation of EWS by PKC—Because EWS contains the conserved PKC phosphorylation site found in other IQ domain proteins, we examined the phosphorylation of EWS by PKC. The phosphorylation of EWS by PKC was dependent on the presence of both Ca$^{2+}$ and phospholipids (Fig. 6A). Furthermore, bisindolylmaleimide, a specific inhibitor of PKC, totally inhibited phosphorylation of EWS. Interestingly, TLS/FUS and PSF were also phosphorylated by PKC in the presence of Ca$^{2+}$ and phospholipids, whereas p54$^{++}$ and P48 were poor PKC substrates (Fig. 7B and data not shown).

An analysis of the kinetics for phosphorylation by PKC showed that phosphorylation of EWS resulted in a mobility shift on SDS-PAGE gels (Fig. 6B). Within 15 min, two bands with equal intensity were apparent on the Coomassie Blue-stained gel, demonstrating that approximately 50% of the protein was phosphorylated. Furthermore, superposition of the autoradiogram with the Coomassie Blue-stained gel showed that only the upper band incorporated phosphate. These fractions were also analyzed by Western analysis using an antibody specific to EWS (EWS 677 Ab) and the IQ Ab (Fig. 6B). Although the EWS 677 Ab recognized both forms of EWS, the IQ Ab only recognized the unphosphorylated form of EWS. Furthermore, the peptide corresponding to the IQ domain of EWS phosphorylation was reduced by increasing the presence of Ca$^{2+}$, but this interaction was apparently weaker. Since PKC phosphorylation of neuromodulin or neuromodulin is inhibited by CaM, we monitored the effect of CaM on EWS phosphorylation. If EWS binds CaM through its IQ domain, CaM should also inhibit phosphorylation of EWS by PKC. PKC phosphorylation of EWS was reduced by increasing concentrations of CaM with maximal inhibition at approximately 4.5 μM CaM (Fig. 7B and C). Calmodulin also inhibited PKC phosphorylation of PSF and TLS/FUS but did not affect autophosphorylation of PKC (data not shown). These data are consistent with the hypothesis that the major PKC phosphorylation site of EWS is within the IQ domain, which includes or overlaps with the CaM binding domain.

PKC Phosphorylation Inhibits Binding of RNA to EWS—To...
A protein fraction was carried out for 15 min at 30 °C. CaM was added to Lanes Type B, 2 μg) and calcineurin (lanes CN, 0.5 μg) were resolved by 8% SDS-PAGE, stained with Coomassie Blue, or transferred to a polyvinylidene difluoride membrane. Blots were incubated with biotinylated CaM in the presence of 1 mM CaCl₂ or 5 mM EGTA. Bound calmodulin was detected with alkaline phosphatase-conjugated avidin. The autoradiogram shows the position of Coomassie Blue-stained EWS, TLS/FUS, and P48. B, CaM inhibits phosphorylation of EWS, TLS/FUS, and P48. C, CaM inhibits phosphorylation of EWS, TLS/FUS, and PSF by PKC. PKC phosphorylation of type A protein fraction was carried out for 15 min at 30 °C. CaM was added at a final concentration of 0 (lane a), 0.525 (lane b), 1.05 (lane c), 2.01 (lane d), and 4.2 μM (lane e). Phosphoproteins were separated by 8% SDS-PAGE and visualized by autoradiography. C, the autoradiogram was analyzed by densitometry, and phosphorylation is presented as a percentage of control phosphorylation (lane a in panel B). Shown is a representative of three separate experiments.

To determine whether phosphorylation of EWS modulates RNA binding, we first investigated the effect of soluble RNA homopolymers on the phosphorylation of EWS, PSF, and TLS/FUS. Increasing concentrations of all RNA homopolymers inhibited the phosphorylation of all three proteins by PKC (Fig. 8A). Poly(U) was the strongest inhibitor of EWS phosphorylation (Fig. 8B). The RNA homopolymers did not affect phosphorylation of the GST-neurogranin fusion protein or the auto-phosphorylation of PKC (data not shown).

To determine whether phosphorylation of EWS by PKC affects RNA binding, proteins from the type A fraction were phosphorylated by PKC for 1 h in the presence or absence of bisindolylmaleimide, the PKC inhibitor. Phosphorylation of the type A fraction for 1 h induced a complete mobility shift of EWS on SDS gels (Fig. 9A, part a), indicating that it was totally phosphorylated by PKC. PKC phosphorylation was blocked by bisindolylmaleimide (Fig. 9A, part d). The same amount of protein was incubated with poly(G)- or poly(U)-Sepharose beads, which were then washed with 2 M urea in the presence of 50, 100, or 200 mM NaCl. Proteins bound to the poly(U)- or poly(G)-Sepharose beads were separated by SDS-PAGE (Fig. 9A, parts b and c), and phosphoproteins were detected after autoradiography (Fig. 9A, parts e and f). EWS had comparable affinity for poly(G) and poly(U) (Fig. 9A, parts b and c), whereas TLS/FUS had a stronger affinity for poly(G) and PSF for poly(U).

When EWS was phosphorylated by PKC (Fig. 9A, –bisInd) its affinity for poly(G) and poly(U) was significantly reduced compared with the unphosphorylated protein (+bisInd). This was particularly evident when interactions between poly(U) and phosphorylated or unphosphorylated EWS were compared with increasing salt concentration. At 100 mM NaCl, binding of phosphorylated EWS to poly(U)-Sepharose was completely inhibited, but unphosphorylated EWS was still absorbed (Fig. 9A, part c). Interactions between the RNA homopolymers and PSF as well as TLS/FUS were also lowered by PKC phosphorylation. Phosphorylated forms of EWS and PSF apparently have a lower affinity for poly(U) than do the unphosphorylated forms of these proteins. Phosphorylation of EWS, PSF, and TLS/FUS was also examined when these proteins were absorbed to RNA homopolymers (Fig. 9B). PKC phosphorylation of EWS, TLS/FUS, and PSF was significantly reduced when they were first bound to poly(U)-Sepharose beads. However, phosphorylation of p54nrb was not affected by poly(U)-Sepharose beads. This suggests that there are two distinct interaction sites for poly(U) and poly(G) on these proteins and that poly U masks the phosphorylation site(s) of PKC. Alternatively, poly(U) binding may induce a conformational change that affects the ability of PKC to phosphorylate PSF, EWS, and TLS/FUS. Collectively, these data suggest that PKC phosphorylation of EWS and interactions with RNA may be mutually exclusive.

DISCUSSION

The rapid redistribution of Ca²⁺ throughout intracellular compartments, including the nucleus, is a general signaling mechanism for transfer of information conveyed by growth factors and hormones. Several nuclear events including DNA replication, DNA repair, and cell cycle progression are regulated by Ca²⁺ through the activation and nuclear translocation of CaM or PKC isoforms (51–58).

Since the IQ domain may be a general regulatory element for Ca²⁺ signal transduction, it was important to determine if the nucleus contains IQ domain proteins other than P68-RNA helicase (26). In this study we show that the nuclear RNA-binding protein EWS contains an IQ domain and that its phosphorylation by PKC inhibits RNA binding. In addition, EWS co-purifies with several other RNA-binding proteins that are also PKC substrates.

Sequence analysis indicates that EWS shares four common amino acids with the IQ domain of neurogranin, neuromodulin, and P68-RNA helicase, the residues 284–QXSFR. Since the IQ Aβ immunoreacted only with the unphosphorylated form of EWS, the site of PKC phosphorylation is very likely Ser²⁶⁶, which is within the IQ domain. Ser²⁶⁶ corresponds to the PKC phosphorylation sites in neurogranin and neurogranin (2, 6).

Furthermore, EWS contains Phe²⁶⁷ and Arg²⁶⁸, two amino acids adjacent to Ser²⁶⁶ that are conserved in the IQ domain of neurogranin, neuromodulin, and P68-RNA helicase. Substitution of these residues with other amino acids greatly diminishes phosphorylation of neurogranin by PKC (4). Collectively, these data indicate that EWS contains an IQ domain with Ser²⁶⁶ acting as the primary site for PKC phosphorylation.

The EWS IQ domain is located in the C-terminal end of the N-terminal domain of EWS (Fig. 10), which contains multiple
copies of the sequence SYGQQS (32). The N-terminal domain was first described as the EWS domain that fuses with the DNA-binding domain of the ETS transcription factor FLi-1 after chromosomal translocation in Ewing’s sarcoma (32). Depending on the breakpoint positions of genes coding for EWS or FLi-1, three different types of chimeric oncoproteins are produced (32, 59). The IQ domain is lost in type 1 and type 2 chimeric oncoproteins, whereas it is retained in type 3 EWS/FLi-1. The N-terminal domain acts as a transcriptional activator when it fuses with the Fli-1 DNA-binding domain of Fli-1 (60–62). The fusion protein mediates the transformation activity of EWS/FLi-1, suggesting that the N-terminal domain interacts with the basal transcription machinery through protein-protein interactions (62, 63). It would be interesting to determine if PKC or CaM regulate transcription and transformation activities of the type 3 EWS/FLi-1 through its IQ domain.

The inhibition of RNA binding to EWS by PKC phosphorylation is consistent with other data in the literature indicating that interactions between RNA and RNA-binding proteins are regulated by phosphorylation. For example, a dynamic phosphorylation/dephosphorylation cycle of C hnRNP protein modulates its binding to pre-mRNA (64). Recently, Municio et al. (49) discovered that the phosphorylation of hnRNPA1 by PKC-ζ severely impairs its ability to bind to RNA through its RNA-binding consensus sequence. PKC phosphorylation of the IRPs RNA-binding proteins also regulates their affinity for RNA (65). Although we did not determine the mechanism for PKC inhibition of RNA binding to EWS, it is probably not due to direct phosphorylation of the RNA interaction site. Using truncated forms of EWS-b, an EWS spliced form (Fig. 10), Ohno et al. (48) showed that the full-length EWS-b binds to poly(G) and poly(U) homopolymers, whereas the C-terminal domain (CTD) of EWS-b only binds to poly(G) homopolymers. The N-terminal domain of EWS contains the PKC phosphorylation site, and it has no RNA binding activity of its own. Consequently, inhibition of RNA binding by PKC is not due to direct phosphorylation of the RNA binding site. PKC inhibition of EWS binding to RNA may reflect an indirect role of the N-terminal domain in regulating RNA binding.

Several lines of evidence classify EWS as a member of the hnRNP family, proteins that are associated with hnRNAs (including pre-mRNA) and are implicated in RNA metabolism (66, 67). EWS shares structural homology with hnRNP proteins (Fig. 10) including the consensus RNA-binding domain RBD/RRM/RNP-CS flanked with auxiliary RNA-binding RGG boxes.
Fig. 9. Phosphorylation of EWS by PKC inhibits binding to poly(U)- or poly(G)-Sepharose. A, PKC phosphorylation of the type A fraction (5 µg) was carried out in the presence or absence of bisindolylmaleimide (parts a and d, lanes Type A ± bisInd.) for 1 h at 30 °C. The reaction was stopped by the addition of urea at a final concentration of 2 M. Samples were then washed in a solution containing 20 mM Hepes, pH 7.2, 5 mM MgCl₂, 1.5 mM EGTA, and 50, 100, or 200 mM NaCl as indicated. The drained beads were boiled for 5 min in Laemmli buffer (40 µl). Samples were subjected to SDS-PAGE, stained with Coomassie Blue (lanes Poly U) or poly(G)-Sepharose beads (lanes Poly G) in buffer A containing 50 mM NaCl for 1 h at 4 °C. Beads were washed in the same urea buffer and were then washed in a solution containing 20 mM Hepes, pH 7.2, 5 mM MgCl₂, 2.5 mM CaCl₂, and 5 mM DTT. Phosphorylation was carried out by the addition of purified PKC, a mixture of phosphatidylinositol-serine (100 µg/ml) and diolein (10 µg/ml), and [γ-32P]ATP (500 µM, 0.1 mCi/ml) for 30 min at 30 °C. Finally, beads were washed in urea buffer, drained, and boiled in 50 µl of Laemmlli loading buffer. Samples were analyzed by SDS-PAGE, stained with Coomassie Blue (left panel), dried, and autoradiographed (right panel). The position of EWS and the co-purified proteins are shown. A large sized 8% SDS-PAGE was used in the post-transcriptional modifications of pre-mRNA.

Inhibition of EWS RNA Binding by PKC Phosphorylation

well as EWS and other RNA-binding proteins whose interactions with RNA may be regulated by PKC. Our data support the general hypothesis that Ca²⁺-signal transduction pathways may control the activity of RNA-binding proteins through PKC or CaM. The presence of IQ domains in two RNA-binding proteins, EWS and p68 RNA helicase, raises the interesting possibility that this domain may coordinate general regulation of RNA processing by PKC or CaM.

Acknowledgments—We thank Dr. B. Murphy for useful advice in peptide mapping and A. Calvin for the sequencing experiments. We thank Dr. J. Campbell for help in PKC purification and for useful comments during this work. We thank Dr. D. Ron and Dr. J. G. Patton for the gift of the RNS/LTS antibody and αPSF antibody, respectively. We also thank Dr. A. R. Krainer for the gift of p54nrb recombinant protein and PRP18 antibody. We especially thank Dr. L. P. Baker for comments on this manuscript.

REFERENCES

1. Alexander, K. A., Wakim, B. T., Doyle, G. S., Walsh, K. A., and Storm, D. R. (1988) J. Biol. Chem. 263, 7544–7549
2. Baudier, J., Deloulme, J. C., Van Dersselaer, A., Black, D., and Matthes, H. W. (1991) J. Biol. Chem. 266, 229–237
3. Watson, J. B., Battenberg, E. F., Wong, K. K., Bloom, F. E., and Sutcliffe, J. G. (1996) Biochemistry 35, 2630–2638
4. Chen, S. J., Klann, E., Geuer, M. C., Powell, C. M., Sessoms, J. S., and Sweatt, J. D. (1993) Biochemistry 32, 1032–1039
5. Alexander, K. A., Cimler, B., Meier, K. E., and Storm, D. R. (1987) J. Biol. Chem. 262, 6108–6113
6. Apel, E. D., Byford, M. F., Au, D., Walsh, K. A., and Storm, D. R. (1990) Biochemistry 29, 2330–2336
7. Chapman, E. R., Au, D., Nicolson, T. A., and Storm, D. R. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 37–44
8. Gerendasy, D. D., Herron, S. R., Wong, K. K., Watson, J. B., and Sutcliffe, J. G. (1994) J. Biol. Chem. 269, 22420–22426
9. Gerendasy, D. D., Herron, S. R., Wong, K. K., Watson, J. B., and Sutcliffe, J. G. (1994) J. Mol. Neurosci. 5, 133–148
10. Liu, Y. C., and Storm, D. R. (1990) Trends Pharmacol. Sci. 11, 107–111
11. Gandy, C., Waage, M. C., Allen, R. G., and Baizer, L. (1996) J. Biol. Chem. 271, 26988–26995
12. Chao, S., Benowitz, L. I., Krainc, D., and Irwin, N. (1996) Brain Res. 740, 195–202
13. Takagi, T., and Cox, J. A. (1990) J. Biol. Chem. 265, 19271–19277
14. Nemson, J. R., Morgan, J. I., Fullerton, S. M., Danho, W., Hilbush, B. S., and Wengenack, T. M. (1996) J. Biol. Chem. 271, 15911–15917
15. Mu, F. T., Callaghan, J. M., Steele-Mortimer, O., Stenmark, H., Parton, R. G., Campbell, P. L., McCluskey, J., Yeo, J.-P., Tock, R. F. C., and Tob, B.-H. (1995) J. Biol. Chem. 270, 13503–13511
16. Farnsworth, C. L., Fresney, N. W., Rosen, L. B., Gwathmey, K. E., and Feig, L. A. (1995) Nature 376, 524–527
17. Moseketer, M. S., and Cheney, R. E. (1995) Annu. Rev. Cell. Dev. Biol. 11, 633–675
18. Neil, V. A., and Young, M. W. (1994) Development 120, 2235–2243
19. Weissbach, L., Slinn, J. C., Moomaw, D. M., and Sjostrom, A. J. (1995) Mol. Cell. Biol. 15, 4869–4878
20. Munshi, H. G., Burks, D. J., Joyal, J. L., White, M. F., and Sacks, D. B. (1996) Biochemistry 35, 15883–15889
21. Petrova, T. V., Takagi, T., and Cox, J. A. (1996) J. Biol. Chem. 271, 21095–21099
Inhibition of EWS RNA Binding by PKC Phosphorylation

27377

23. Xie, X., Harrison, D. H., Schlichting, I., Sweet, R. M., Kalabokis, V. N., Szentgyorgyi, A. G., and Cohen, C. (1994) Nature 366, 306–312
24. Shue, F. S., Azmitia, E. C., Marshak, D. R., Parker, P. J., and Rottenberg, A. (1994) Mol. Brain Res. 21, 62–66
25. Shue, P. F., Huang, F. L., and Huang, K. P. (1995) Arch. Biochem. Biophys. 316, 335–342
26. Buelt, M. K., Glidden, B. J., and Storm, D. R. (1994) J. Biol. Chem. 269, 25967–25970
27. Schmid, S. R., and Linder, P. (1992) J. Biol. Chem. 267, 252–256
28. Bonser, R., Faura, M., Serratos, J., Renau-Piqueras, J., Fruschi, M., and Bachs, O. (1995) Mol. Cell. Biol. 15, 661–670
29. Munico, M. M., Lozano, J., Sanchez, P., Moscat, J., and Diaz-Meco, M. T. (1995) J. Biol. Chem. 270, 15884–15891
30. Idries, H., Kumar, A., Casas Finet, J. R., Guo, H., Damuni, Z., and Wilson, S. H. (1994) Biochemistry 33, 11382–11390
31. Bachs, O., and Carafoli, E. (1992) J. Biol. Chem. 267, 10786–10790
32. Delattre, O., Zucman, J., Plougastel, D., Desmace, C., Melot, T., Peter, M., Kvar, H., Joubert, I., de Jong, P., Rouleau, G. A., and Thomas, G. (1992) Nature 359, 661–666
33. Triche, T. J. (1997) Encyclopedia of Cancer 1, 652–659
34. Crozat, A., Aman, P., Mandahl, N., and Ron, D. (1993) Nature 363, 640–644
35. Rabbitts, T. H., Forster, A., Larson, R., and Nathan, P. (1993) Nat. Genet. 4, 175–180
36. Calvin, C., Neubauer, G., Mann, M., and Lamond, A. I. (1995) RNA 1, 724–733
37. Patton, J. G., Porro, E. B., Galceran, J., Tempst, P., and Nadal-Ginard, B. (1993) Genes Dev. 7, 393–406
38. Pinol-Roma, S., Choi, Y. D., Matunis, M. J., and Dreyfuss, G. (1988) Genes Dev. 2, 215–227
39. Cleary, D. W., Fisher, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102–1106
40. Towbin, J., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
41. Laemmli, U. K. (1970) Nature 227, 680–685
42. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
43. Billingsley, M. L., Pennypacker, K. R., Hoover, C. G., Brigati, D. J., and Kinzler, K. L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7585–7589
44. Mattern, K. A., Humble, B. M., Muijers, A. O., de Jong, L., and van Driel, R. (1996) J. Cell. Biochem. 62, 275–289
45. Hirling, H., Scheffner, M., Bestle, T., and Stahl, H. (1989) Nature 339, 562–564
46. Lane, D. P., and Hoeffer, W. K. (1986) Nature 328, 167–170
47. Zinzsnzer, H., Albalat, R., and Ron, D. (1994) Genes Dev. 8, 2513–2526
48. Ohno, T., Ouchida, M., Lee, L., Gatalica, Z., Rao, V. N., and Reddy, E. S. P. (1994) Oncogene 9, 3087–3097
49. Gehani, P., Patton, J. G., and Reed, R. (1994) EMBO J. 13, 3356–3367
50. Dong, B., Hara, S. D., Kobayashi, R., and Krainer, A. R. (1993) Nucleic Acids Res. 21, 4085–4092
51. Bachs, O., Agell, N., and Carafoli, E. (1992) Biochim. Biophys. Acta 1113, 259–270
52. Irvine, R. F., and Divecha, N. (1992) Semin. Cell Biol. 3, 225–235
53. Bachs, O., Agell, N., and Carafoli, E. (1994) Cell Calcium 16, 289–296
54. Divecha, N., Bandic, H., and Irvine, R. F. (1994) Cell Calcium 16, 297–300
55. Hepler, P. K. (1994) Cell Calcium 16, 322–330
56. Lin, C., Hanjinecky, G., and Thomas, A. P. (1994) Cell Calcium 16, 247–258
57. Nicotera, P., Zhivotovsky, B., and Orrenius, S. (1994) Cell Calcium 16, 279–288
58. Buchner, K. (1995) Eur. J. Biochem. 238, 211–221
59. Zucman, J., Melot, T., Desmazes, C., Ghysdael, J., Plougastel, B., Peter, M., Zucker, J., Triche, T., Ambros, P., Combarel, V., Lenoir, G., Auras, A., Thomas, G., and Delattre, O. (1993) EMBO J. 12, 4481–4487
60. Ohno, T., Rao, V. N., and Reddy, E. S. (1993) Cancer Res. 53, 5559–5563
61. May, W. A., Lessnick, S. L., Grahn, B. S., Klemm, M., Lewis, B. C., Lundford, L. B., Hromas, R., and Denny, C. T. (1993) Mol. Cell. Biol. 13, 7397–7398
62. Lessnick, S. L., Grahn, B. S., Denny, C. T., and May, W. A. (1993) Oncogene 10, 423–431
63. May, W. A., Gishizky, M. L., Lessnick, S. L., Lundford, L. B., Lewis, B. C., Delattre, O., Zucman, J., Thomas, G., and Denny, C. T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5752–5756
64. Mayrand, S. H., Dwen, P., and Pederson, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7764–7768
65. Schalinsky, K. L., and Eisenstein, R. S. (1996) J. Biol. Chem. 271, 1168–1176
66. Dreyfuss, G., Matunis, M. J., Pinol-Roma, S., and Burd, C. C. (1993) Annu. Rev. Biochem. 62, 289–321
67. Weighardt, F., Biaumonti, G., and Riva, S. (1996) BioEssays 18, 747–756
68. Burd, C. C., and Dreyfuss, G. (1994) Science 265, 615–621
69. Getzenberg, R. H. (1994) J. Cell. Biochem. 55, 22–31
70. van Driel, R., Wansink, D. G., van Steensel, B., Grande, M. A., Schul, W., and Kiledjian, D. J. (1992) J. Histochem. Cytochem. 40, 151–159
71. Kiledjian, D. J., and Dreyfuss, G. (1992) EMBO J. 11, 2655–2664
72. Zini, N., Martelli, A. M., Neri, L. M., Bavelloni, A., Sabatelli, P., Santi, S., and Maraldi, N. M. (1993) Histochem. Cell Biol. 103, 447–457
73. Serratos, J., Pujol, M. J., Bachs, O., and Carafoli, E. (1986) Biochem. Biophys. Res. Commun. 150, 1162–1169