Modulatory effect of the transmembrane domain of the protein-tyrosine kinase encoded by oncogene \textit{ros}: Biological function and substrate interaction

CONG S. ZONG AND LU-HAI WANG

Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029

Communicated by Hidesaburo Hanafusa, July 25, 1994 (received for review June 9, 1994)

ABSTRACT There is a 3-aa insertion in the transmembrane (TM) domain of the \textit{p68} protein-tyrosine kinase encoded by avian sarcoma virus UR2 \textit{v-ros} as compared with that of the protooncogene \textit{c-ros}. The effect of this insertion on biological function and biochemical properties of \textit{v-ros} protein was investigated by deleting these 3 aa to generate the mutant TM1. This mutant has greatly reduced transforming, mitogenic, and tumorigenic activities despite the fact that the protein-tyrosine kinase activity and cell-surface localization of TM1 protein are unaffected. However, unlike \textit{v-ros}, mutant TM1 protein becomes glycosylated, is differentially phosphorylated, and fails to induce tyrosine phosphorylation of a 88-kDa protein and a major substrate of insulin receptor, insulin receptor substrate 1. The TM1 protein is unable to associate with phosphatidylinositol 3-kinase and fails to promote association of insulin receptor substrate 1 with phosphatidylinositol 3-kinase. By contrast, tyrosine phosphorylation of Shc protein and phospholipase C \textit{Y} as well as interaction of Grb2 protein with Shc and SOS protein signaling components are unaltered in the TM1 infected cells. Our results show that the TM-domain sequence of \textit{p68} profoundly affects its function and substrate interaction. The mutant defines a signaling pathway including phosphatidylinositol 3-kinase, insulin receptor substrate 1, and possibly an 88-kDa protein that does not overlap the Ras pathway and is important for full transforming and mitogenic potency of \textit{v-ros} protein-tyrosine kinase.

Protooncogene \textit{c-ros} codes for a receptor protein-tyrosine kinase (PTK) (1) with sequence and structural hierarchy closely resembling those of the sevenless protein of \textit{Drosophila} (2-5). In addition, the PTK domain of \textit{Ros} shares a greater homology with those of insulin receptor and insulin-like growth factor I receptor (IGFR) than any other PTK family members (3-7). The ligand of \textit{c-Ros} remains unknown; however, \textit{c-Ros} is specifically expressed in the epithelial cells of kidney tubules and villi of intestine and has been suggested to operate in their development and mature function (3-5, 8, 9). Spontaneous transduction of \textit{c-ros} by an avian leukemia virus resulted in its structural alterations and oncogenic activation (10). The oncogene \textit{v-ros} carried in the genome of avian sarcoma virus UR2 differs from \textit{c-ros} structurally in three aspects (11): (i) all 1873 aa of the extracellular domain of \textit{c-Ros} except the last 6 aa are truncated in \textit{v-ros}, and the remaining C-terminal c-Ros is joined at its N-terminus to retroviral Gag sequence to form an in-frame Gag–Ros fusion protein, (ii) the receptor (Ser-Leu-Thr) insertion in the middle of the transmembrane (TM) domain of \textit{v-ros} in comparison with that of \textit{c-Ros}, and (iii) there is a small C-terminal deletion and fusion of c-Ros to viral Env sequence to generate the C-terminal end of \textit{v-ros}. There are no mutations in \textit{v-ros} other than these three changes. \textit{v-ros} codes for a 68-kDa TM Gag–Ros fusion PTK with the Gag portion protruding extracellularly (12). Both Gag and TM domains are essential for the transforming function of \textit{p68} (13, 14).

Our recent study (11) showed that the 3' change of \textit{v-ros} has little effect on activation of the transforming potential of \textit{c-ros}, whereas 5' truncation (deleting all but 6 aa upstream of the TM domain) and fusion to \textit{gag} are required for activating \textit{c-ros} transforming activity, although this activity is not as potent as that of \textit{v-ros}. Combination of the 5' alteration and the 3-aa insertion in the TM domain is sufficient to convert \textit{v-ros} into an oncogene as potent as \textit{v-ros} (11). This observation suggests that the 3-aa insertion in the TM domain of \textit{v-Ros} may be important to its function.

To further inquire into the role of the 3-aa insertion of \textit{v-Ros}, we deleted this sequence and investigated the transforming function and signal transduction of the mutant protein. Our results indicate that the 3-aa deletion has no effect on PTK activity of \textit{v-Ros}, but this deletion does result in greatly reduced transforming and mitogenic potency, as well as altered protein modifications and substrate interaction despite the fact that the cytoplasmic domain of the mutant protein is unaltered. Thus, the mutant allows us to identify certain cellular proteins and a signaling pathway that may be important in cell transformation. This mutant also shows that specificity of the \textit{v-Ros} PTK-substrate interaction is not solely determined by its cytoplasmic domain.

MATERIALS AND METHODS

Cells, Viruses, Biological Assays, and DNA Transfection. The preparation of chicken embryo fibroblasts (CEF) and colony assay of virus-infected CEF were as described (15). Molecular clones and their derived viruses of UR2, UR2AV, CC5d (a \textit{c-ros}-derived virus), VC (a \textit{v-ros} \textit{x c-ros} recombinant), SRC×ROS (a \textit{src} \textit{x c-ros} recombinant), NMI (encoding a Gag–IGFR fusion protein), and UIIGFR (encoding the full-length human IGFR) have been described (10, 11, 16, 17). Transfection of DNA into CEF by the calcium phosphate or Polybrene method was as described (11, 14).

Antibodies. Anti-phosphotyrosine (a-P-Tyr) antibodies (Abs) Py20 and RC20, anti-phospholipase C \textit{Y} (PLC \textit{Y}), and anti-Grb2 were purchased from Transduction Laboratories (Lexington, KY). The polyclonal a-P-Tyr serum GAPY1 and the anti-Ros serum have been described (16). Anti-Shc and anti-phosphatidylinositol 3-kinase (a-P3-kinase) were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-SOS was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal Ab, 5C2, against a P-Tyr protein from UR2-transformed CEF was obtained by using a method similar to that of ref. 18 (S. M. Jong, J. Chan, T.

Abbreviations: Ab, antibody; IRS1, insulin receptor substrate 1; PTK, protein-tyrosine kinase; PLC \textit{Y}, phospholipase C \textit{Y}; P3-kinase, phosphatidylinositol 3-kinase; TM, transmembrane; CEF, chicken embryo fibroblasts; IGFR, insulin growth factor 1 receptor.
Fig. 1. Genomic structure of UR2 and TM1 viruses. Construction of TM1 has been described. The TM1 DNA differs from UR2 DNA (10) only by the lack of Ser-Leu-Thr (SLT) insertion beginning at the 17th amino acid of the 29-aa TM domain of UR2 p6889-ros.

Moran, and L.-H.W., unpublished work). Rabbit anti-insulin receptor substrate 1 (α-IRS1) serum was raised against a fusion polypeptide of glutathione transferase and the C-terminal 272 aa of rat IRS1 (19).

Protein Analysis. [35S]Methionine or [32P]P, labeling of cells and protein extraction were as described (11-14). In vitro PTK assay, PI3-kinase assay, immunoanalysis of tyrosine-phosphorylated total or specific proteins, and bior-in-labeling of surface proteins have been described (11-14, 17). Inhibition of glycosylation by tunicamycin treatment was done by described procedures (11). For inhibition of Golgi function by brefeldin A (20, 21), CEF were incubated with the inhibitor (1 μg/ml) for 2 hr before [35S]methionine labeling; this incubation continued over a 4 hr-labeling period. Possible oligomerization of Ros proteins under non-denaturation condition in polyacrylamide gels and by glycerol gradient sedimentation was analyzed by described methods (11, 17, 22). Tryptic and V8 protease peptide mappings of in vitro or metabolically labeled Ros proteins were done as reported (12, 23).

RESULTS

Construction of TM1 Mutant. CC5d is a weak transforming virus containing the 5' truncated c-ros fused at its 5' end to gag at the position identical to that of UR2 gag-ros (11). The only differences between p6889-ros fusion proteins of UR2 and CC5d are the 3-aal insertion in the TM domain and the C-terminal change in UR2 protein. A BamHI-Mam I DNA fragment of 410 bp containing the TM domain was prepared from CC5d DNA and used to replace the corresponding region of UR2 DNA. The DNA fragment was sequenced and confirmed as identical to that of UR2 except for absence of the 3-aal insertion. The resulting virus, TM1, is identical to UR2 virus except for the 3-aal absence in the TM domain (Fig. 1).

Transforming, Mitogenic, and Tumorigenic Activities of TM1 Mutant. TM1 and UR2 DNAs were individually transfected together with UR2AV DNA into CEF for comparison of their mitogenic and transforming activities. TM1 has a markedly reduced transforming activity, as judged by morphological alteration and colony-forming ability of the transfected CEF (Fig. 2). On average, there is a 4-fold reduction in numbers of colonies from TM1-infected cells. Although the TM1-infected cells grew at a rate 3- to 4-fold faster than normal CEF, they are 2- to 3-fold slower than the UR2 cells when plated at 1 x 10⁶ cells per 6-cm dish and maintained in medium with 1% chicken serum and either 5 or 0.5% calf serum (data not shown). Equivalent colony-forming units (2 x 10⁶) of viruses TM1 and UR2 were injected into wing webs of newborn chicks. Ten days after injection, most chicks from both groups developed areas of redness or tiny tumors near the injection sites. In 3 weeks, all UR2-infected chickens developed tumors (average size, 3.5 cm³), whereas the tumors of TM1-infected chickens remained small (average size, <1 cm³).

Analysis of TM1 Protein. To study the biochemical basis for the reduced transforming and mitogenic activities of TM1, various properties of its encoded protein were investigated. Fig. 3A shows that the TM1 protein has an in vitro PTK activity indistinguishable from that of UR2 and is tyrosine phosphorylated similarly in infected cells. TM1 protein is also detected on the cell surface as abundantly as UR2 protein (Fig. 3B). Like UR2 p6889-ros, TM1 protein is not dimerized, as reflected by unaltered gel mobility with or without denaturation by 2-mercaptoethanol (Fig. 3C). Under the same conditions, the NM1 Gag-IGFR protein existed as a monomer after denaturation and as dimers without denaturation, even with iodoacetamide, which prevented artificial oligomerization during protein extraction, confirming our previous observation (17). In addition, possible formation of protein complex was also investigated by glycerol gradient sedimentation of proteins extracted with Nonidet P-40 lysate buffer without denaturing agents. No evidence of protein complex formation was seen for either UR2 or TM1 p6889-ros (data not shown).

During analysis, we noticed that the TM1 protein appeared heterogeneously as multiple bands (Fig. 3). We suspected that this might reflect differential phosphorylation and/or glycosylation. Fig. 4A shows that the upper bands of TM1 proteins disappeared upon tunicamycin treatment, suggesting that glycosylation was most likely responsible for the observed heterogeneity. Brefeldin A, an inhibitor of protein transport to Golgi apparatus and further modification in this organelle (20, 21) also affected appearance of the TM1 protein but not of the UR2 protein (Fig. 4C). Glycosylation of the TM1 protein was further supported by digestion with V-glycosidase F, which affected TM1 protein and IGFR but not the UR2 protein (Fig. 4B). When CC5d and CV5d proteins, both lacking the 3-aal insertion in the TM domain, were examined, similar results with tunicamycin and brefeldin A were seen (data not shown). These data indicate that absence of the 3-aal insertion in the TM domain allows glycosylation of the TM1 protein. Conversely, the presence of such amino acids prevents glycosylation of UR2 protein.

We next examined ability of the TM1 protein to phosphorylate cellular substrates. Fig. 5A shows that overall, the TM1 p6889-ros is as efficient as that of UR2 in phosphorylating cellular proteins. However, a tyrosine-phosphorylated (P-
Tyr) 88-kDa protein is missing in the TM1-infected CEF; this was more clearly seen with the polyclonal a-P-Tyr serum GapY1 (Fig. 5B). A monoclonal Ab 5C2 (Fig. 5C), which we recently prepared against the P-Tyr proteins from UR2-transformed CEF, recognized an 88-kDa protein that was tyrosine-phosphorylated in the UR2-infected, but not in the TM1-infected, cells (Fig. 5C), and this band comigrated with the 88-kDa protein band detected in the total protein immunoblot described above. The apparently stronger phosphorylation of a 38-kDa protein in TM1 cells was not reproducible (compare Fig. 5 A and B).

We reported (24) that the p68PAP-ros could associate with PI3-kinase; we compared this property in TM1 and UR2 proteins. Fig. 6A shows that TM1 protein failed to associate with PI3-kinase. Because the PTK domain of v-Ros shares a high homology with that of insulin receptor and PI3-kinase can be activated by the major substrate of insulin receptor, IRS1 (25), we examined the ability of TM1 protein to phosphorylate IRS1 and promote its association with PI3-kinase. Fig. 6 shows that unlike in UR2-transformed CEF, IRS1 was not appreciably tyrosine-phosphorylated and associated with PI3-kinase in the TM1-infected cells.

We also examined the interaction of the Ros proteins with other signaling components, including PLCγ and those in the Ras pathway—such as Shc, Grb2, and SOS protein (26, 27). Fig. 7 shows that the extent of tyrosine phosphorylation of Shc and PLCγ was similar in TM1- and UR2-infected cells. In addition, immunoprecipitation of UR2- and TM1-infected cell lysates with anti-Shc or anti-Sos followed by immunoblotting with anti-Grb2 revealed indistinguishable interaction between Grb2 and Shc or Sos (data not shown). Similar immunoprecipitation with anti-Grb2 followed by immunoblotting with a-P-Tyr Ab RC20 also did not differ noticeably in Grb2-associated P-Tyr protein patterns in the two virus-infected cultures (data not shown).

Except for the difference in glycosylation, TM1 protein appears indistinguishable from UR2 protein in various aspects, including expression level, PTK activity, extent of autophosphorylation, status of protein complex, and cell-surface localization. To explore the basis for the observed differential substrate interaction of TM1 protein, we looked for a possible difference in its sites of phosphorylation. The UR2 and TM1 p68PAP-ros labeled with 32P metabolically or by in vitro kinase reaction were subjected to V8 protease or tryptic mapping. The V8 protease cleavage pattern of the in vitro autophosphorylated TM1 protein, labeled exclusively at tyrosine sites, is identical to that of the UR2 protein, except that a small peptide fragment is missing among the cleavage products of TM1 protein (Fig. 8). The tryptic peptides of metabolically [32P]P-labeled TM1 protein resemble those of the UR2 protein, except for the retarded chromatographic mobility for one tryptic peptide spot and altered ratios of phosphorylation for two others (data not shown). The V8 protease and tryptic mappings strongly suggest that at least one tyrosine site is involved in those differences. Possibly those differences in phosphorylation are responsible for the altered substrate interaction of TM1 protein.

**DISCUSSION**

Our results show that deletion of a 3 aa insertion in the middle of the TM domain of virus UR2 p68PAP-ros profoundly affects its substrate interaction and biological activity. The deletion also results in different post-translational modifications of the TM1 protein, including glycosylation and phosphorylation.

**Fig. 3.** Analysis of UR2 and TM1 proteins. (A) PTK activity. One milligram of protein extracts from control or viral DNA-transfected CEF was immunoprecipitated with anti-Ros; precipitates were divided into triplicates and subjected to an in vitro kinase reaction (Upper) and immunoblotting with a-P-Tyr Ab RC20 (Middle), or anti-Ros (Lower). (B) Surface protein labeling. In a parallel culture of TM1 and UR2, intact cells were labeled with biotin; total cellular proteins were extracted, immunoprecipitated with anti-Ros, and analyzed by immunoblotting with an avidin–alkaline phosphatase conjugate. The src × ros recombinant virus encoding a membrane-associated cytoplasmic protein (16) was included as control to show the validity of surface protein labeling. Only TM1-3 was compared here. UR2 and TM1 proteins are indicated by an arrow. Identity of the diffuse bands below the viral proteins is unknown. (C) Analysis of possible dimerization of UR2 and TM1 proteins. Proteins were extracted and immunoprecipitated as in A, except that 10 μM of iodoacetamide was added to extraction buffer to prevent artificial dimerization. Washed immunoprecipitates were resuspended in sample buffer with or without 5% (vol/vol) 2-mercaptoethanol for PAGE as described (17). β-mercap., 2-mercaptoethanol.

**Fig. 4.** Glycosylation of TM1 protein. (A) Control and virus-infected cells were metabolically labeled with [35S]methionine for 4 hr in medium without tunicamycn (10 μg/ml). Proteins were extracted with RIPA buffer (12, 22), immunoprecipitated with anti-Ros, and analyzed. The 78-kDa chaperon protein known as Bip protein was reproducibly induced upon tunicamycin (Tunic.) treatment (11). (B) Anti-Ros immunoprecipitates of [35S]methionine-labeled proteins in A were resuspended in 20 μl of 1% SDS and boiled for 2 min; then 180 μl of phosphate-buffered saline/20 mM sodium azide/10 mM EDTA/50 mM Nonidet P-40 was added, and the mixture was boiled again for 2 min; one unit of N-glycosidase F was added to one of the duplicates, and the mixture was incubated at 37°C for 15 hr. Digested proteins were analyzed by gel electrophoresis similarly. (C) [35S]Methionine labeling and protein analysis were done as in A, except that brefeldin (1 μg/ml) was used instead of tunicamycn. Only TM1-3 was analyzed in B and C.
FIG. 5. Phosphorylation of cellular proteins by UR2 and TM1 proteins. Total cellular proteins were extracted and immunoblotted with a-P-Tyr Ab RC20 (A) or Ab GAPY1 (B). Arrow, ~88-kDa protein band that is tyrosine-phosphorylated in UR2-infected, but not in TM1-infected, cells. Only TM1-3 was analyzed in B. (C) Proteins were extracted with RIPA buffer, immunoprecipitated (IP) with Ab 5C2, divided into duplicates, and immunoblotted (West) with either Ab RC20 (Upper) or Ab5C2 (Lower). C, control.

The different effects of brefeldin A, which perturbs protein transport from endoplasmic reticulum to Golgi apparatus and subsequent modifications there (20, 21), imply that posttranslational trafficking of TM1 and UR2 proteins may also differ. A c-ros-containing virus called CC and a c-ros x v-ros recombinant called CV, as well as their transforming variants called CC5d and CV5d, respectively, all code for Gag-Ros fusion proteins without the 3aa insertion in the TM domain, and they are all glycosylated (11). Therefore, the insertion appears to perturb the Ros protein structure and thus prevent it from being recognized by glycosylating enzymes during the initial stages of protein translocation and modification in endoplasmic reticulum. There are numerous reports on the effect of mutations of amino acids surrounding TM domains.

FIG. 7. Tyrosine phosphorylation of Shc and PLCγ. (A) Equivalent amounts (500 μg each) of total cellular protein extracts were immunoprecipitated with anti-PLCγ and analyzed by immunoblotting with Ab RC20 or anti-PLCγ. Identity of doublet bands of ~95 kDa is unknown. (B) Cellular protein extracts (500 μg each) from control or various virus-infected CEF were immunoprecipitated with anti-Shc and analyzed by immunoblotting with Ab RC20. ts, Temperature-sensitive.

FIG. 6. Interaction of Ros protein with PI3-kinase and IRS1. (A) Equivalent amounts (600 μg each) of cellular extracts were immunoprecipitated with anti-IRS1 or anti-Ros. Anti-IRS1 and an aliquot of the anti-Ros immunoprecipitate were assayed for PI3-kinase activity (Upper and Middle). An equal aliquot of the anti-Ros precipitate was analyzed by immunoblotting with anti-Ros (Lower). (B) Total cellular extracts (600 μg each) from control, TM1-3-infected, or temperature-sensitive (ts) UR2 mutant-infected CEF kept at 35°C or 41°C were immunoprecipitated with anti-IRS1, followed by immunoblotting (West.) with Ab RC20. In a separate experiment, anti-IRS1 precipitates from TM1-3- or UR2-infected CEF were analyzed by immunoblotting with anti-IRS1. Arrow, IRS1 band.

FIG. 8. V8 protease mapping of phosphorylated UR2 and TM1 proteins. TM1-3 and UR2 proteins were immunoprecipitated by anti-Ros and subjected to an in vitro autokinase reaction in the presence of [γ-32P]ATP. 32P-labeled viral proteins were separated in polyacrylamide gel, excised, and digested with V8 protease at the indicated concentrations and analyzed by electrophoresis. Arrows, peptide fragments differentiating UR2 protein from TM1 protein.
and deletions within the domains of various proteins with respect to their posttranslational modifications, membrane anchorage, orientation, and trafficking (28). However, to our knowledge, the observation that a small amino acid insertion in the middle of the TM domain causes differential glycosylation has not been previously reported. A single Val → Glu mutation in the TM domain of c-Neu, an epidermal growth factor receptor-related PTK, is responsible for its activation of cell transforming and tumorigenic potential (29). The mutation apparently locks the Neu protein in a stable dimerized state, mimicking that of ligand binding, and activates PTK activity and autophosphorylation (30–32). The mutation was also reported to increase the turnover rate of Neu protein and render it differentially phosphorylated at certain Thr/Ser residues and refractive to inhibition by protein kinase C (33, 34). By contrast, two studies indicated that insulin receptor and epidermal growth factor receptor were relatively refractive to mutations within their TM domains, including the similar Val → Glu mutation (35, 36). None of those studies examined the possible differential substrate interaction of those mutant receptor PTKs.

The valuable class of transformation-defective mutants of the PTK-containing oncogenic viruses are those that retain kinase activity because they could help identify the important substrates for mitogenesis and cell transformation. Our data show that mutation in TM1 protein has no effect on its PTK activity, autophosphorylation, and surface localization. However, TM1 protein interacts differently with a number of cellular proteins—including PI3-kinase, IRS1, and an 88-kDa protein despite the fact that its cytoplasmic domain is identical to that of UR2 protein. Therefore, this result appears an exception to the hypothesis that the specificity of substrate recognition is determined solely by the cytoplasmic domain of a receptor PTK, as indicated by studies of chimeric receptors (26, 27). Because tyrosine phosphorylation of Shc protein and interaction among SOS, Shc, and Grb2 proteins were unaltered, our results suggest that the Ras signaling pathway is insufficient to confer full transforming activity to Ros PTK. The PI3-kinase and IRS1-related signaling pathway appears complementary to the Ras pathway and is needed for full transforming and mitogenic potency. Therefore, the TM1 mutant may have helped delineate two nonoverlapping pathways required for full biological activity of v-Ros protein. Despite its activation by a variety of PTKs, the function of PI3-kinase remains obscure. Similarly, the nature and function of the 88-kDa protein remain to be elucidated. The 88-kDa protein is distinguishable from the protein recognized by a monoclonal Ab against chicken cortactin (data not shown). At present, we do not know how PI3-kinase and the 88-kDa protein are involved in the biological activity of v-Ros protein.

The mechanism for the observed differential substrate interaction is unclear. Insertion of 3 aa in the TM domain of UR2 p68MG400 may cause conformational change of the TM peptide spanning the membrane and alter interaction with certain membrane protein(s) which, in turn, affects conformation of the cytoplasmic domain, changing phosphorylation and substrate interaction. Alternatively, the insertion may cause protrusion of some amino acids into the cytoplasmic domain, directly affecting its configuration. It was reported that a point mutation in the domain of T-cell antigen receptor β chain caused its failure to assemble with CD3 complex (37).

Peculiarly, although the Gag–IGFR fusion protein forms dimers on cell surface, Gag–Ros does not (29). In NMI1-encoded Gag–IGFR, Gag is linked directly to the TM domain of IGFR (17). In p68MG400, Gag is linked to Ros at a position 6 aa upstream of the TM domain (10). We suspect that the determinants for those fusion proteins exist either as dimers or as single polypeptides on the cell surface that probably reside in the TM and/or cytoplasmic domains. Both an earlier study (22) and our current study failed to detect dimerization of p68MG400. Although dimerization may be important for Gag–IGFR function, it appears unimportant for Gag–Ros. However, whether monomeric Gag–IGFR is functional remains to be seen. Study of chimeric recombinants between Gag–Ros and Gag–IGFR should resolve those questions.

We thank Dr. T. Parsons for the anti-cortactin Ab. This work was supported by National Institutes of Health Grant CA29339.