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Primary structure, expression, and signal-dependent tyrosine phosphorylation of a *Drosophila* homolog of extracellular signal-regulated kinase

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ABSTRACT **The extracellular signal-regulated kinases (ERKs) comprise a class of protein-serine/threonine kinases that are activated in response to a wide variety of extracellular signals transduced via receptor tyrosine kinases. Activation of the ERKs requires both threonine and tyrosine phosphorylation suggestive of a key role in mediating intracellular events in response to extracellular cues. To critically assess the role of ERKs in intracellular signaling, a genetically tractable receptor tyrosine kinase system would be invaluable. In this paper we report the identification of a *Drosophila* homolog of ERK1 and -2, designated DmERK-A. DmERK-A is 80% identical to rat ERK1 and -2 and is rapidly phosphorylated on tyrosine in response to an extracellular signal activating a receptor tyrosine kinase. Biochemical and histological studies reveal its expression in the eye imaginal disc. These studies provide a first step in a genetic analysis of ERK function.**

The extracellular signal-regulated kinases (ERKs), also known as the mitogen-activated protein (MAP) kinases, are thought to play pivotal roles in the regulation of cellular proliferation and differentiation in response to extracellular signals. ERK1 and ERK2 from rat have been shown to be activated in response to a wide range of factors (e.g., epidermal growth factor, nerve growth factor, and insulin) promoting both cell division and differentiation (1–3). These kinases have also been shown to be activated via the *N*-methyl-D-aspartate receptor, suggesting a role in the process of long-term potentiation (4). There is evidence that one function of ERK1 and/or ERK2 may be the phosphorylation and activation of c-Jun (5) and c-Myc (6), two DNA-binding proteins required for the proper regulation of cellular proliferation and differentiation (7). Despite its proposed importance the requirement for ERK function in any signal-transduction system has not been demonstrated. Analysis of proteins similar to the ERKs in the yeast *Saccharomyces cerevisiae*, KSS1 (8) and FUS3 (9), which are required for cellular response to mating factor, provides genetic evidence that related members of this class are required in developmentally important signal-transduction cascades.

Two receptor tyrosine kinases (RTKs) have been shown to play important roles in the development of the *Drosophila* compound eye and to be highly amenable to genetic analysis. The *Drosophila* homolog of the epidermal growth factor receptor (DER) plays a key role in an early patterning event (10), perhaps in regulating cellular proliferation as in mammalian systems (11). The activation of the sevenless (sev) RTK by the ligand bride-of-sevenless is required for the determination of the R7 photoreceptor neuron (12, 13). Genetic screens for loci that participate in signal-transduction cascades initiated by sev and DER have identified two proteins that act downstream of these RTKs; Dras1 (14) and Son-of-sevenless (Sos) (14–16). Sos is related in sequence to a guanine nucleotide-binding regulatory protein of *S. cerevisiae*, CDC25 (17, 18), and thus may directly positively regulate the activity of Dras1. In addition *Gap1* (19), a gene that negatively regulates R7 development, encodes a homolog of both the mammalian GTPase-activating protein GAP (20) and the *IRA* gene products of *S. cerevisiae* (21). Both GAP and IRA antagonize Ras activation.

An alternative approach to the elegant suppressor/enhancer screens used to identify components of these two RTK signal-transduction cascades is to use molecular techniques to identify homologs of molecules activated by RTKs in other systems that are expressed in the developing eye of *Drosophila*. The ERKs are unique in this regard. First, in every RTK system analyzed to date, rapid (1–5 min) activation of the ERKs has been reported (1). Second, the ERKs are two of only a handful of enzymes whose activity has been shown to be altered by tyrosine phosphorylation; ERK activation requires phosphorylation of both tyrosine and threonine residues (22). And finally, in PC12 rat pheochromocytoma cells the phosphorylation, and thus activation, of ERK1 and -2 is dependent upon Ras activation (23). These data allow the placement of ERK1 and -2 downstream of not only the RTKs, but also of Ras, and suggest that in *Drosophila* Dras1 may regulate the activity of a *Drosophila* homolog of ERK1 and -2 in response to extracellular cues that regulate development of the compound eye. We have identified a *Drosophila melanogaster* homolog of ERK1 and -2, which we have designated DmERK-A.§

MATERIALS AND METHODS

Materials. *Taq* DNA polymerase was purchased from Perkins–Elmer/Cetus, and Superscript reverse transcriptase from Bethesda Research Laboratories. PCR and sequencing primers were synthesized by Operon Technologies, Alameda, CA. All other enzymes and vectors were obtained from Boehringer Mannheim, Promega, Stratagene, or New England Biolabs. Bovine insulin was from Collaborative Research. The 4G10 anti-phosphotyrosine monoclonal antibody was from Upstate Biotechnical, Lake Placid, NY. The ECL (enhanced chemiluminescence) reagent system was obtained from Amersham.

Reverse Transcription–Polymerase Chain Reaction (RT–PCR). The sense primers were 5′-GGGAATTCGGMGARGGMACNTAYGG-3′ (amino acid sequence GEGAYG) and 5′-GGGAATTCGGMGARGGMGCNTAYGG-3′ (amino acid sequence GEGTYG). The *Eco*RI site included for clon-

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Abbreviations: ERK, extracellular signal-regulated kinase; RTK, receptor tyrosine kinase; RT–PCR, reverse transcription–polymerase chain reaction.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M95124).

ing purposes is underlined. The antisense primers were 5'-TTTCTAGAGGNGCNCGRTACCANCGNGT-3' (amino acid sequence TRWYRAP) and 5'-TTTCTAGAGGNGCNCGRTACCANAGNGT-3' (amino acid sequence TLWYRAP). The *Xba* I site included for cloning purposes is underlined. Degenerate nucleotide positions are indicated: M = A/C, N = G/A/T/C, R = A/G, Y = C/T. RT–PCR was carried out (24) using 5 μg of total RNA isolated from *Drosophila* third-instar imaginal discs. The RT–PCR products were isolated and cloned into pBluescript SK (Stratagene) by utilizing *Eco*RI and *Xba* I sites incorporated into the sense and antisense primers, respectively.

cDNA Cloning and Sequencing. A *D. melanogaster* embryonic plasmid cDNA library (pNB40, 4- to 8-hr embryos) was screened with isolated cloned PCR products as described (25). PCR and cDNA clones were sequenced on both strands with the Sequenase 2.0 kit (United States Biochemical) by the dideoxy chain-termination method.

Polyclonal Antibodies and Immunohistochemistry. Rabbit polyclonal antibodies were raised to bovine serum albumin-conjugated synthetic peptides. The DmERK-A-(347–364) peptide (ENDDISRDALKSLIFEET) was selected based on the low number of amino acid identities to the corresponding regions of the deduced group 2 protein. Peptide conjugation and antibody affinity purification were carried out as described (26). Immunohistochemistry was carried out (13) with affinity-purified DmERK-A antibodies at a dilution of 1:200.

Cell Culture. *Drosophila* S2 cells were normally maintained as a semi-adherent culture in Schneider's medium with 10% fetal bovine serum.

Western Blot Analysis. Tissue samples (≈100 embryos, 50 eye discs, 4 heads, and 2 bodies) were homogenized in 2× Laemmli sample buffer and heated at 100°C for 5 min. Samples were cleared by centrifugation. Supernatants were electrophoresed in SDS/10% polyacrylamide gels, and the proteins were transferred to nitrocellulose by semi-dry blotting. Primary and secondary antibody incubations were carried out as described (13). Blots were visualized with the ECL system and Kodak X-Omat film.

Immunoprecipitation and Tyrosine Phosphorylation of DmERK-A. Approximately 5×10^6 cells were placed in Schneider's medium without added serum and cultured for 24–48 hr. This medium was then removed and replaced with medium containing no supplement (unstimulated) or 150 nM bovine insulin (stimulated). After incubation for 5 min the medium was removed and the cells were lysed in 1 ml as described (26). All solutions used during the immunoprecipitation contained sodium fluoride, sodium orthovanadate, and sodium molybdate as phosphatase inhibitors. DmERK-A was immunoprecipitated using 25 μl of affinity-purified DmERK-A antibodies covalently coupled to protein A-agarose (26). The immunoprecipitated DmERK-A was released from the beads by a 5-min treatment with 25 μl of 100 mM triethylamine and processed for Western blot analysis (26). The Western blots were probed with the 4G10 anti-phosphotyrosine monoclonal antibody (1:1000). In all experiments the amount of DmERK-A in the immunoprecipitates was determined by probing a blot with the affinity-purified DmERK-A antibodies (1:2000) to ensure that differences in phosphotyrosine content observed were not due to variability in the amount of DmERK-A that was immunoprecipitated. The specificity of the 4G10 antibody was assessed by the addition of either phosphotyrosine (16 μM) or a mixture of phosphoserine (400 μM) and phosphothreonine (400 μM) to 4G10/Western blot incubation mixture.

RESULTS AND DISCUSSION

RT–PCR Identification of ERK-Related Sequences. Using the sequences of rat ERK1 and -2 (2, 3), and yeast kinases KSS1 (8) and FUS3 (9), we set out to identify homologs of the ERKs expressed during the development of the compound eye. Alignment of the proposed amino acid sequences of these kinases revealed several extended stretches of amino acid identity. Two of these, GEGAYG and TRWYRAP, were found to be of sufficient length and specificity to allow for the design of degenerate oligonucleotide primers for use in PCR amplification. The corresponding primers were used to amplify sequences present in total RNA isolated from *Drosophila* imaginal discs. A single major amplification product of ≈500 base pairs was cloned and individual cloned fragments were sequenced. Twenty-two clones, of the 123 clones examined, were highly homologous to protein kinases (Fig. 1) (27). The 22 clones fell into four groups based on sequence identity. (*i*) Group 1 and 2 clones were found to be 83% and 44% identical, respectively, to both ERK1 and ERK2 of rat. (*ii*) Group 3 clones were 82% identical to the human galactosyltransferase-associated kinase p58/GTA (28). Overexpression of the p58/GTA kinase has been shown to cause cell cycle arrest early in the G_1 phase of the cell cycle (28). (*iii*) Group 4 clones were most closely related to the CDC28/cdc2 class of proteins (65% identity) (29, 30). The full-length sequence corresponding to group 4 was found to be identical to the cdc2 cognate gene of *D. melanogaster* (31, 32).

Cloning of Group 1 cDNA. Group 1 clones were selected for further study because of their remarkable similarity to rat ERK1 and -2. A 2.6-kilobase cDNA was isolated from a *Drosophila* embryonic cDNA library by using a group 1 cDNA as a probe and was sequenced (Fig. 2). An alignment of the open reading frame with the sequences of rat ERK1 and -2 and yeast FUS3 and KSS1 revealed several extended regions of amino acid identity (Fig. 3). The identified open reading frame of 376 amino acids is the most similar to rat ERK1 and -2 over its entire length [80% identical (297/375)] (Fig. 4). Two recently identified ERK-related kinases from *Xenopus laevis*, Xp42 (33) and MPK1 (34), also show the same high degree of identity to rat ERK1 and -2. We refer to the protein encoded by this 2.6-kilobase cDNA as DmERK-A. Cytological localization using salivary gland polytene chromosomes localized the DmERK-A gene to a single site at 45A on the second chromosome (data not shown).

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                          II                 III           IV                                  V
GROUP 1   MVVSADDTLTNQRVAIKKIS-PFEHQTYCQRTLREI-TILTRFKHENIIDIRDILRVD----SIDQMRDVYI-VQCLMETDLYKLLKTQR
GROUP 2   QVSKAVVRGTNMHVAIKKLARPFQSAVHAKRTYREL-RLLRHMDHENVIGLLDIFHPHPANGSLENFQQVYLLVTHLMDADLNNIIRMQQ
GROUP 3   VVYRAKDKRTNEIEALKRLKMEKEKEGFPITSRREINTLLKGGQHPNIVTVREIVVGSNMDKIFIVMDYVEHDLKSLMETMKNRKQSFFP
GROUP 4   IVYKARTNSTGQDVALKKIRLEGETEGVPSTAIREI-SLLKNLKHPNVVQLFDVVIS--GNNLYMIFEYLNMDLKKLM--DKKK-DVFTP

                           VI                    VII                     VIII
GROUP 1   -LSNDHICYFL-YQILRGLKYIHSANVLHRDLKPSNLLNKT--CDLKICDFGLARIADPEHDHTGFLTEYVA
GROUP 2   HLSDDHVQ-FLVYQILRGLKYIHSA-VIHRDLKPSNIAVNNEDCELRILDFGLAR-P-TE-NE---MTGYVA
GROUP 3   --GE--VKC-L-TQQLRAVAHLH-DNILHRDLKTSNLLLSHKGI-LKVGDFGLAR----EYGSPIKKTSLVV
GROUP 4   QL----IKSYM-HQILDAVAFCHTNRILHRDLKPQNLLVDTAG-KIKLADFGLAR-AFNVPMRA-Y-THEVV
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FIG. 1. ERK/cdc2-related kinases expressed in developing imaginal discs. Shown is an alignment of the deduced amino acid sequences of RT–PCR products obtained by using primers specific for the ERK/cdc2 class of proteins. Sequence analysis placed 22 of 123 RT–PCR products cloned into one of four groups (groups 1–4) based on sequence identity (see text). Roman numerals above the sequence denote kinase subdomains (27). The alignment was assigned by inspection.

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TTAAGTTATAGACTTTTGGTTAAATTCATAATAAAGTAAATAAAGATTTTGCACTTATTGCTGTTCACTTAAATAAGATAAAGTGGGACATATTCAGTTGTAAAAGTGTTATTACAATC   120
CACGCATACATACATTGCGAATTTTGCATAGACGGTGTATTTTGTATCATAACATCTTTAGTAGTATTTCAAGTTTTCCAGAAGAAACGTTAGTC ATG GAG GAA TTT AAT TCG   270
                                                                                                MET GLU GLU PHE ASN SER     6

AGC GGA TCA GTA GTT AAT GGT ACA GGA TCT ACG GAA GTT CCT CAA TCT AAT GCT GAA GTT ATA AGG GGA CAA ATA TTT GAA GTT GGT CCT   360
SER GLY SER VAL VAL ASN GLY THR GLY SER THR GLU VAL PRO GLN SER ASN ALA GLU VAL ILE ARG GLY GLN ILE PHE GLU VAL GLY PRO    36

AGG TAT ATT AAA CTC GCC TAT ATA GGT GAA GGA GCT TAT GGC ATG GTT GTG TCT GCG GAT GAC ACG CTA ACA AAC CAA AGA GTT GCA ATA   450
ARG TYR ILE LYS LEU ALA TYR ILE GLY GLU GLY ALA TYR GLY MET VAL VAL SER ALA ASP ASP THR LEU THR ASN GLN ARG VAL ALA ILE    66

AAA AAA ATA TCG CCC TTT GAA CAC CAA ACT TAT TGT CAA AGG ACT CTC AGA GAA ATA ACC ATA TTG ACC AGA TTT AAA CAT GAA AAC ATT   540
LYS LYS ILE SER PRO PHE GLU HIS GLN THR TYR CYS GLN ARG THR LEU ARG GLU ILE THR ILE LEU THR ARG PHE LYS HIS GLU ASN ILE    96

ATT GAT ATT CGA GAT ATT CTT CGA GTT GAT AGC ATA GAC CAA ATG AGA GAT GTT TAT ATT GTA CAG TGT TTG ATG GAG ACT GAT TTG TAT   630
ILE ASP ILE ARG ASP ILE LEU ARG VAL ASP SER ILE ASP GLN MET ARG ASP VAL TYR ILE VAL GLN CYS LEU MET GLU THR ASP LEU TYR   126

AAA CTA CTT AAA ACA CAG AGG CTA AGT AAT GAT CAC ATC TGT TAC TTC TTA TAT CAG ATA TTG CGT GGA CTC AAG TAC ATT CAT TCC GCA   720
LYS LEU LEU LYS THR GLN ARG LEU SER ASN ASP HIS ILE CYS TYR PHE LEU TYR GLN ILE LEU ARG GLY LEU LYS TYR ILE HIS SER ALA   156

AAC GTC TTG CAT CGG GAC CTT AAG CCA AGT AAT TTA CTG TTG AAC AAG ACG TGC GAC TTA AAA ATT TGC GAC TTT GGA TTG GCT CGT ATT   810
ASN VAL LEU HIS ARG ASP LEU LYS PRO SER ASN LEU LEU LEU ASN LYS THR CYS ASP LEU LYS ILE CYS ASP PHE GLY LEU ALA ARG ILE   186

GCA GAT CCC GAG CAC GAT CAT ACT GGC TTT CTC ACA GAA TAC GTT GCT ACC CGA TGG TAT AGA GCA CCT GAA ATA ATG CTT AAC TCA AAA   900
ALA ASP PRO GLU HIS ASP HIS THR GLY PHE LEU THR GLU TYR VAL ALA THR ARG TRP TYR ARG ALA PRO GLU ILE MET LEU ASN SER LYS   216

GGA TAC ACC AAA TCT ATA GAC ATA TGG TCC GTT GGC TGC ATT TTG GCT GAA ATG TTA AGT AAT CGG CCA ATA TTT CCT GGA AAA CAT TAC   990
GLY TYR THR LYS SER ILE ASP ILE TRP SER VAL GLY CYS ILE LEU ALA GLU MET LEU SER ASN ARG PRO ILE PHE PRO GLY LYS HIS TYR   246

CTG GAT CAA CTT AAT CAT ATT CTT GGA GTC TTG GGT TCA CCG TCC CGG GAC GAT TTA GAG TGT ATT ATT AAT GAA AAG GCA CGG AAC TAT  1080
LEU ASP GLN LEU ASN HIS ILE LEU GLY VAL LEU GLY SER PRO SER ARG ASP ASP LEU GLU CYS ILE ILE ASN GLU LYS ALA ARG ASN TYR   276

TTG GAA TCT TTA CCA TTT AAG CCA AAT GTA CCC TGG GCG AAA CTA TTT CCA AAT GCT GAT GCG TTG GCT TTA GAT CTC CTT GGA AAA ATG  1170
LEU GLU SER LEU PRO PHE LYS PRO ASN VAL PRO TRP ALA LYS LEU PHE PRO ASN ALA ASP ALA LEU ALA LEU ASP LEU LEU GLY LYS MET   306

TTA ACA TTT AAC CCG CAT AAA CGG ATT CCT GTC GAG GAA GCT CTT GCA CAT CCC TAT TTA GAG CAA TAT TAT GAT CCT GGA GAT GAG CCT  1260
LEU THR PHE ASN PRO HIS LYS ARG ILE PRO VAL GLU GLU ALA LEU ALA HIS PRO TYR LEU GLU GLN TYR TYR ASP PRO GLY ASP GLU PRO   336

GTC GCT GAA GTG CCA TTT CGG ATT AAT ATG GAA AAT GAT GAC ATT TCT CGA GAT GCC CTG AAG TCG CTC ATT TTT GAA GAA ACC TTA AAA  1350
VAL ALA GLU VAL PRO PHE ARG ILE ASN MET GLU ASN ASP ASP ILE SER ARG ASP ALA LEU LYS SER LEU ILE PHE GLU GLU THR LEU LYS   366

TTT AAG GAA CGA CAA CCA GAC AAT GCG CCT TAA GAATGCGCTGTAAGCTTTAAGAATTTGAGTATATAGCAAAACAATTGCTCCACTTCTCCATTTAGCATAATTTTT  1458
PHE LYS GLU ARG GLN PRO ASP ASN ALA PRO OPA                                                                                 376
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FIG. 2. Nucleotide and deduced amino acid sequence of DmERK-A.

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                                                          I                        II                 III              IV
DmERK-A            MEEFSSGSVVNGTGSTEVPQSNAEVIRGQIFEVGPRYIKLAYIGEGAYGMVVSADDTLTNQRVAIKKISPFEHQTYCQRTLREITILTRFK-HENIIDIR    100
rERK-1                       EPRGTAGVVP:VPGEV::VK:QP:D::::TQ:Q:::::::::S::Y:HVRKT:::::::::::::::::::::Q::LG:R-:::V:G::     92
rERK-2                                 MAAAAAAGP:MVK::V:::::::N:S:::::::::C::Y:NLNKV:::::::::::::::::::::K::L::R-:::::::N     85
XP42                       MAAAAASSN:GGGP:MV:::A:D::::TN:S:::::::::C::HCNINKV:::::::::::::::::::::K::L:::-::::G:N     90
MPK1                       MAAAGAASN:GGGP:MV:::A:D:::::N:::::::::::C::H:NVNKV:::::::::::::::::::::K::L:::-::::G:N     90
FUS3                                          MPKRIVYNISSDFQLKSLL:::::V:C::THKP:GEI::::::E::DKPLFAL:::::K::KH::-::::T:F     75
KSS1                                          MARTIT:DIPSQ:KLVDL::::::T:C::IHKPSGIK:::::Q::SKKLFVT::I:::KL:RY:HE::::S:L     76

                      V                                        VI                   VII
DmERK-A   DILRVDSIDQMRDVYIVQCLMETDLYKLLKTQ-----RLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLLNKTCDLKICDFGLARIADPEHD-------HT    194
rERK-1    ::::APTLEA:::::::D::::::::::S:-----Q:::::::::::::::::::::::::::::::::::::T::::::::::::::::::::::-------::    185
rERK-2    ::I::PT:E::K:::::D::::::::::::::::-----H::::::::::::::::::::::::::::::::::T::::::::::::V:::D::--------::    178
XP42      ::I:APT:E::K:::::D::::::::::::::::-----H::::::::::::::::::::::::::::::::::T::::::::::::V:::D::--------::    184
MPK1      ::I:APT:E::K:::::D::::::::::::::::-----H::::::::::::::::::::::::::::::::::T::::::::::::V:::D::--------::    184
FUS3      N:Q:P::FENFNE:::I:E::Q:::HRVIS::-----M::D:::Q::I::T::AV:VL:GS::I:::::::::I:SN::::V::::::::I:ESAADNSEPTGQQ    175
KSS1      :KV:PV:I:KLNA::L:EE::::::Q:VINN:NSGFST::D::VQ::T:::::A::S::::Q:I:::I:::::::::SN::::V:::::::CLASSS:---SRETLV    178

    VIII                         IX                              X
DmERK-A  GFLTEYVATRWYARPEIMLNSKGYTKSIDIWSVGCILAEMLSNRPIFPGKHYLDQLNHILGVLGSP-SRDDLECIINEKARNYLESLPFKPNVPWAKLFPNAD--ALA    299
rERK-1   :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::I:::::-:QE::N::::M::::::Q:::S:TK:A::::::KS:--SK:    289
rERK-2   :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::I:::::-:QE::N::::L::::::L:::H:NK:::NR:::::::--SK:    282
XP42     :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::-:QE::N::::L::::::L:::H:NK:::NR:::::::--PK:    289
MPK1     :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::-:QE::N::::L::::::L:::H:NK:::NR:::::::--PK:    289
FUS3     SGM::::::::::::V::T:AK:SRAM:V::C::::::LFLR::::::RD:RH::LL:F:II:T:H:DN::R::ESPR::E:IK:::MY:AA:LE:M::RVN--PKG    280
KSS1     ::M::::::::::::::::TFQE::TAM::::C:::::::V:GK:L:::RD:HH::WL::EV::T:-:FE:FNQ:KSKR:KE:IAN::MR:PL::ETVWSKT:LNPDM    285

                  XI
DmERK-A  LDLLGKMLTFNPNKRIPVEEALAHPYLEQYYDPGDEPVAEV------------PFRINMENDDISRDALKSLIFEETLKFKERQPDNAP                    376
rERK-1   ::::DR::::::::::T:::::::::::::::T::::::E------------::TFD::L::LPKER::E:::Q::AR:QPGA:-E::                     367
rERK-2   ::::D::::::H:::E::Q::::::::::::::S:::I::A------------::KFD::L::LPKEK::E::::::AR:QPGYR-S                      258
XP42     ::::D::::::H:::E:::::::::::::::::S::::::A------------::KFE::L::LPKET::E::::::AR:QPGY                         361
MPK1     ::::D::::::H:::E:::::::::::::::::S::::::A------------::KFE::L::LPKET::E::::::AR:QPGY                         361
FUS3     I:::Q:::V:D:A:::TAK:::E::::QT:H::N:::EG:PIPPSF--------:EFDHHKEALTTKD::K::WN:IFS                              353
KSS1     I:::DK::Q:::D:::SAA:::R::::AM:H::S:::EYPPLNLDDEFWKLDNKIMRPE:EEEV:IEM::DMLYD:LMKTM:                           368
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FIG. 3. Comparison of amino acid sequences of ERK-related proteins. Amino acid sequences of DmERK-A, rat ERK1 (2) and ERK2 (3), *Xenopus laevis* Xp42 (33) and MPK1 (34), and the yeast proteins KSS1 (7) and FUS3 (8) were aligned by using Geneworks 2.0 (IntelliGenetics). The algorithm employed is similar to FASTA and utilizes a PAM-250 scoring matrix. Identical amino acids are indicated by a colon, and gaps by a hyphen (-). Roman numerals above the sequence denote the kinase subdomains (27).

	DmERK-A	rERK-1	rERK-2	Xp42	MPK1	FUS3	KSS1
DmERK-A	100	79	80	79	80	47	47
rERK-1		100	83	82	83	45	43
rERK-2			100	95	95	48	50
Xp42				100	98	46	44
MPK1					100	46	44
FUS3						100	51
KSS1							100

FIG. 4. Percent amino acid identities among the ERK family of protein kinases. Identity between each pair of proteins was determined over the length of the shorter partner. Gaps in the sequence alignment were not included in the percent identity presented.

Expression of DmERK-A During Development. To study the pattern of DmERK-A expression in the developing eye disc and to facilitate biochemical studies, polyclonal antibodies were raised to specific regions of DmERK-A. The immunizing peptides were selected so that they would distinguish between DmERK-A and related sequences present in group 2; antibodies purified by peptide affinity chromatography were specific for DmERK-A, failing to recognize group 2 sequences (Fig. 5*A*). The DmERK-A antibodies recognized a single band at ≈44 kDa from embryos, third-instar eye imaginal discs, and adult tissue (Fig. 5*B*), as well as the *Drosophila* S2 embryonic cell line (data not shown). This band is consistent with the size of DmERK-A predicted from the sequence of the cDNA and is similar to that of rat ERK1 and -2. The possibility cannot be excluded, however, that the purified DmERK-A antibodies also recognize a second, closely related protein as is seen with antisera raised against rat ERK1 and ERK2 (35). Note, however, that rat ERK1 and ERK2 are easily distinguished by their apparent molecular weight on SDS/PAGE analysis. Immunohistochemical analysis using light microscopy revealed DmERK-A expression in all cells of the developing eye imaginal disc (Fig. 5*C*). Immunolocalization demonstrated that DmERK-A could be found in both the nucleus and the cytoplasm of S2 cells (data not shown). In ≈10% of stained S2 cells, however, DmERK-A appeared to be excluded from the nucleus.

Tyrosine Phosphorylation of DmERK-A. To address one aspect of the functional relatedness of DmERK-A and the mammalian ERKs, we tested whether DmERK-A is phosphorylated on tyrosine in response to an extracellular ligand acting through its receptor on the surface of the S2 cells. S2 cells express a saturable high-affinity receptor that recognizes insulin from several mammalian species (36). This receptor, like its mammalian counterpart, possesses an intrinsic protein-tyrosine kinase activity (37). DmERK-A was immunoprecipitated from both insulin-treated and untreated S2 cells, and the level of tyrosine phosphorylation was determined by probing Western blots with an antibody to phosphotyrosine. Following insulin treatment of S2 cells DmERK-A showed a rapid, transient, and marked increase in phosphotyrosine content relative to that in unstimulated cells (Fig. 6). Maximal stimulation was observed at 4 min after insulin addition and returned to baseline levels by 8 min. Anti-phosphotyrosine antibody immunoreactivity was selectively blocked by free phosphotyrosine (16 μM) but not by phosphoserine (400 μM) or phosphothreonine (400 μM). These data indicate that DmERK-A is rapidly phosphorylated on tyrosine via stimulation of the *Drosophila* insulin receptor. The kinetics of tyrosine phosphorylation in the S2 cells in response to insulin are very similar to those observed for rat ERK1 and -2 in mammalian cell lines. In the mammalian systems tyrosine phosphorylation of ERK1 and -2

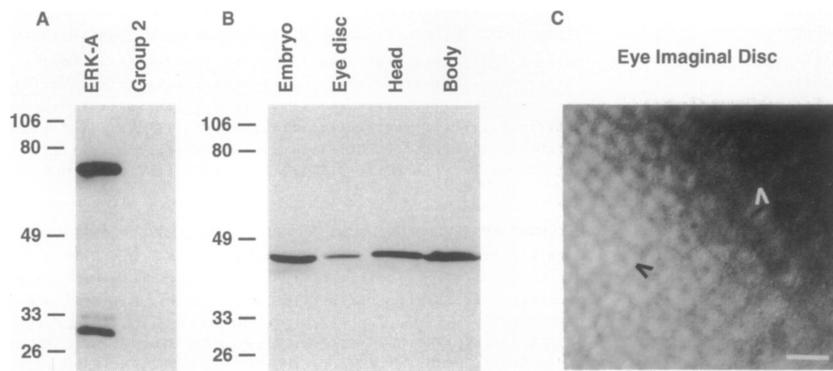

FIG. 5. Spatiotemporal pattern of DmERK-A expression during development. (*A*) The DmERK-A antibodies specifically recognized the DmERK-A/TrpE fusion protein and not the group 2/TrpE fusion. Equal amounts (60 ng) of a bacterial extract containing the respective fusion proteins were loaded in each lane. The molecular mass of the upper band is consistent with the expected size of the DmERK-A/TrpE fusion. The lower band is most likely due to degradation of the full-length product. The group 2 fusion protein is recognized by a group 2-specific antibody (data not shown). Positions of molecular size markers (kDa) are indicated at left. (*B*) Western blot analysis demonstrates that the affinity-purified DmERK-A antibodies recognize a single band at ≈44 kDa that is found at different stages of development. Tissues examined were: 0- to 12-hr embryos, third-instar eye imaginal disc, adult head, and adult body. (*C*) Developing third-instar eye imaginal disc stained with affinity-purified DmERK-A antibodies. Staining is blocked by the peptide to which DmERK-A antiserum was raised (data not shown). An optical section through the disc at the level of the nucleus (black arrowhead) shows an apparent exclusion of DmERK-A from the nuclei of the differentiated photoreceptor cells. Since this is a whole mount preparation, a section below the nuclei can be seen in the same disc (white arrowhead). Nuclei in this preparation were identified by Nomarski optics. DmERK-A is present in the cytoplasm of the photoreceptor cells. Anterior is to the right. (Bar = 20 μm.)

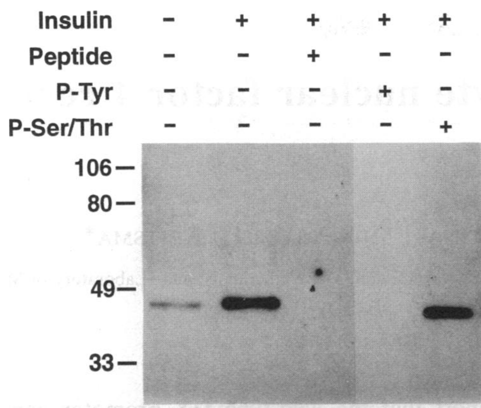

FIG. 6. Signal-dependent tyrosine phosphorylation of DmERK-A. S2 embryonic cells were treated with 150 nM bovine insulin or subjected to mock treatment (control). DmERK-A was immunoprecipitated from both control (−) and insulin-treated (+) cells with affinity-purified DmERK-A antibodies covalently coupled to protein A-agarose. The immunoprecipitate was then subjected to SDS/PAGE and blotted for Western analysis. An anti-phosphotyrosine monoclonal antibody (4G10, 1 μg/ml) detected a single band from the immunoprecipitate, which comigrated with DmERK-A. The recognition of the band could be blocked by the addition of 16 μM (10^3 molar excess) phosphotyrosine (P-Tyr) but not by a mixture of 400 μM (2.5×10^4 molar excess) phosphoserine and 400 μM phosphothreonine (P-Ser/Thr). Addition of the immunizing peptide (Peptide) to the immunoprecipitation mixture (30 μg/ml) blocked the precipitation of DmERK-A.

does not appear to be due to direct phosphorylation by a RTK (38, 39).

Concluding Remarks. Based on the extensive sequence identity between DmERK-A and rat ERK1 and -2, and on the rapid phosphorylation of these proteins in response to an extracellular signal, we propose that the DmERK-A gene of *Drosophila melanogaster* encodes a functional homolog of rat ERK1 and ERK2. ERK activity also appears to be regulated by Ras function in mammalian cells, and Ras function plays a central role in regulating development of the *Drosophila* eye. Interestingly, several proteins that are required for proper eye development, such as rough (40, 41), glass (42), hairy (43), Notch (44), and Sos (14, 16), contain the ERK consensus phosphorylation site Pro-Xaa-(Ser/Thr)-Pro (45–47), suggesting that their functions may be regulated by DmERK-A activity. Our studies provide a biochemical basis for genetic studies investigating the role of DmERK-A in mediating intercellular signaling in the developing eye and for critically assessing its role in signal transduction.

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